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Second Interagency Workshop on In-situ Water-Quality Sensing: Biological Sensors Held at Pensacola Beach, Florida on April 28-30, 1980

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Pensacola Beach, Florida
April 28-30, 1980

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2nd Interagency Workshop on In-Situ Water-Quality Sensing: Biological Sensors

Pensacola Beach, Florida
April 28-30, 1980

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Environmental Protection Agency
National Oceanic and Atmospheric Administration
U.S. Corps of Engineers
U.S. Geological Survey

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FOREWORD

The 2nd Interagency Workshop on In-Situ Water-Quality Sensing convened during the period April 28-30, 1980 in Pensacola Beach, Florida. As the contents of this volume make clear, the need for effective tools for qualifying and quantifying ocean pollution is rapidly becoming critical. The organizers of the workshop hope that this publication will contribute to development of better measurement technology and will receive wide acceptance and careful consideration.

Organizing a conference of this kind is tediously complicated and acknowledgement is especially appropriate for the Steering Committee, responsible for the overall effort. Its members and professional associations are listed on the inside front cover.

Mention should be made that Mr. Arthur Solman of the Water Research Centre of the Medmenham Laboratory at Buckinghamshire came from England to attend and kindly contributed an extemporaneous presentation of their work there. His remarks are included in this conference report.

Acknowledgement is made to Edwin D. Kennedy, NOAA, a member of the Steering Committee who guided this document through the exacting process of editing and putting it all between covers. Finally, the effort in coordinating the conference arrangements and coping with all the myriad details usually attendant to organizing such a conference on-site were most compentently handled by Geraldine Cripe of EPA's Gulf Breeze Laboratory in Pensacola Beach; her contribution is highly appreciated.

A list of the participants in the conference appears at the back of this volume.

A. F. (TONY) MENTINK
1924 - 1980

CHIEF
INSTRUMENTATION DEVELOPMENT BRANCH
ENVIRONMENTAL MONITORING AND SUPPORT LABORATORY
ENVIRONMENTAL PROTECTION AGENCY

DEDICATION

This 2nd Interagency Workshop on In-Situ Water-Quality Sensing is dedicated in memory of the late A. F. (Tony) Mentink, who died suddenly in his EPA laboratory in Cincinnati, Ohio, on January 23, 1980.

At the time of his death, Mr. Mentink was Chief of the Instrumentation Development Branch of the Environmental Monitoring and Support Laboratory of the Environmental Protection Agency. He helped pioneer the development and deployment of automated in-situ water-quality systems, first becoming involved in this activity in the early 1960s. This work included the development of specific pollutant electrodes as well as multiparameter analytical equipment. Mr. Mentink also worked in the field, directing the installation of equipment, which required a strong background in communications technology such as automatic telephony and satellite transmitters as well as the use of computers.

Mr. Mentink was born on April 7, 1923 in Cincinnati and was graduated from the University of Cincinnati in 1952 with a degree in electrical engineering, where he also pursued graduate work. He was elected to the honorary society Eta Kappa Nu.

Mr. Mentink began his professional career as a technical engineer with the General Electric Corporation where he was supervisor of Engineering Analysis in 1961 when he resigned to become a water-quality consultant. Between 1968 and 1972, he was employed as Chief of Instrumentation Development with the Analytical Quality Control Laboratory of the Methods Development and Quality Assurance Research Laboratory. During the period 1965-1976, he published 13 technical papers on the subject of water-quality alone.

As a member of the Institute of Electrical and Electronic Engineers, he was active in a number of its professional groups, including the Professional Group on Electronics, Medicine, and Biology.

In dedicating this conference to Mr. Mentink, the members of the steering committee look back to his active participation in the first such gathering in Las Vegas in 1978, when his contributions were manifold. As development goes forward in this field, his guidance, advice, and technical help will be acutely missed.

Mr. Mentink is survived by his wife Jody and five daughters.

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SUMMARY

The 2nd Interagency Workshop on In-Situ Water-Quality Sensing: Biological Sensors was convened at the Holiday Inn at Pensacola Beach, Florida during the period April 28-30, 1980. The purpose of the workshop was to review the potential of biological mechanisms and organisms as tools for bioassay, measurement and detection of water-quality parameters, with a view toward the development of automated in-situ sensors. The meeting was sponsored by the National Oceanic and Atmospheric Administration, the Environmental Protection Agency, the U.S. Geological Survey, and the U.S. Army Corps of Engineers.

Present capabilities for collection of discrete water-quality information are limited by the lack of adequate in-situ sensing systems. Although advanced measurement systems using satellite and aircraft platforms with remote sensing capabilities are available and information communication networks for the transmission of data over wide areas is commonplace, the usefulness of such systems is limited because real-time or near real-time ground truth verification cannot be obtained. Furthermore, because sensors which directly detect environmental changes of interest are not available, measurements must be carried out through labor-intensive sample collection in the field with attendant deleterious effects such as contamination and in-transit deterioration. The field sampling process must then be followed by tedious analytical procedures in the laboratory. All these factors decrease the quality assurance of the information generated.

The first interagency workshop on automated in-situ sensing was held in February, 1978 in Las Vegas and was sponsored by the Environmental Protection Agency, the National Oceanic and Atmospheric Administration, and the Interagency Work Group on Satellite Data Collection Systems. That workshop concentrated on identification of parameters that must be measured for water-quality assessment and physical and chemical techniques that can be developed for their in-situ detection. This second workshop was planned as part of the continuing interagency effort to improve measurement technology with major emphasis on the biosensing and biological aspects.

A total of 14 papers were presented at the workshop. They covered traditional biological measurements, organism responses as tools for detection, enzymes as tools for detection, and organisms as samplers and bioaccumulators. Paper presentations were followed by four working groups which met concurrently to discuss the state of the art as presented in the papers and formulate recommendations concerning development of these techniques for pollutant and water quality sensing.

Working groups included (1) Biochemical Systems, chaired by Mr. Theodore Major of the Magnavox Corporation; (2) Bioaccumulation, chaired by Dr. Andrew Robertson of NOAA/GLERL; (3) Organism Behavioral Responses, chaired by Mr. Richard W. Paulson, of the U.S. Geological Survey; and (4) Traditional Biological Measurements, chaired by Mr. John B. Bushman of the U.S. Army Corps of Engineers.

In assessing the contextual thrust of the papers offered, the panels evaluated potential capabilities and limitations of biological sensors for improving water-quality measurements; in addition, the groups attempted to identify existing and potential concepts for use in the development of automated in-situ sensors. The panels also considered project development costs and time, and ultimately offered recommendations in terms of priorities related to development planning, taking into consideration the limited resources available and the various needs of the agencies, industries, and universities concerned. Principal conclusions and recommendations are presented in the Integration Panel Report that follows.

In general, the summaries offered by the four panels reflected a marked enthusiasm for the near-future development of practical biosensors for field use. Development of such devices should lead to more reliable and cost-effective water-quality data.

It was also recognized that the U.K. and Europe hold a clear lead over the U.S. in this field of water-quality monitoring and a review of what has been accomplished by the Europeans would be an appropriate initial step in developing a meaningful program for the U.S.

INTEGRATION PANEL REPORT

Composed of the chairmen of the working groups and members of the steering committee, the Integration Panel met at the conclusion of the workshop with the objective of bringing together the diverse work of the four separate panels. There was unanimous agreement among the panel and workshop participants that the emerging technology of biosensing is an exciting new development area as well as a field with great promise in both near- and long-term time frames for providing valuable tools for environmental measurement and assessment in both fresh and marine waters.

One general theme supported the entire Integration Panel discussion: the Federal government has a responsibility to support and foster development of environmental measurement systems, including those involving the use of novel, innovative schemes for providing reliable, cost effective and automated systems where appropriate. Basic research should be carried out at the Federal level in a coordinated fashion to benefit all users, including state and local governments who have neither the funds nor competence to undertake such programs. If reliable devices and measurement schemes can be developed, demonstrated and accepted, particularly by the regulatory agencies, a market sufficient to interest private industry will evolve. The panel also felt it important that the Federal government support development of well documented techniques and procedures as well as hardware, since many environmental measurements are not now and may never be possible through hardware systems alone.

The concept of biosensing is not new. In its simplest form it was employed more than a hundred years ago when deep miners used canaries to detect toxic gases in that underground environment. Little attempt has been made since that time, however, to seriously evaluate general biological concepts for detection of chemical and environmental change.

Biosensors in the form of complete organisms or colonies of organisms can be used as gross integrating detectors for adverse water quality conditions. Analyses of their tissues can provide an integrated measure of the nature of the water in which they have existed. Changes in behavior can indicate stress caused by environmental change. Such techniques have already been demonstrated and, with minimum development effort, some of these techniques could be standardized and automated for routine monitoring. They could be particularly useful as alarms and mechanisms to trigger more directed and sensitive sampling and analysis.

Biosensors can also be developed from parts or components of organisms to perform as detectors of a more specific nature. The natural selectivity that is present in certain components of some organisms can be harnessed to perform as single function detectors for specific chemicals or classes of compounds.

Near Term Development

The panel found that several biosensing schemes were further developed than anticipated. Modest development efforts toward standardization and automation, where possible, could provide operational systems within a very short time.

- Behavioral changes in fish or other whole organisms can be used as alarms and triggering mechanisms, thus initiating intelligent sampling and control actions. The use of fish to monitor industrial effluent is currently well established in Great Britain. Although similar techniques have been demonstrated in the U.S., they are not yet generally accepted. Other organisms could also be used. The behavioral responses of bivalves, for example, could be exploited to indicate changes in the environment in which they reside; bivalves would be particularly sensitive to changes occurring at the water-sediment interface. Some investigations have begun in this area on marine scallops; however, fresh water bivalves present a longer term development problem because methods for their laboratory rearing are not yet well developed.

- Organisms can also be used as integrating samplers. It has long been known that many organisms preferentially accumulate various materials in their tissues, a factor that has been responsible for considerable economic loss over time because of the inability to harvest shellfish from polluted estuaries. On the positive side, however, organisms such as mussels and oysters have been used to collect cumulative samples of heavy metals, radionuclides, and other toxic substances for trend monitoring. The analysis of fresh water periphyton biomass has been used to provide gross information leading to location of pollutant "hot spots."

With these illustrations in mind, it might be useful to propose a system that couples the triggering function of organism behavioral response with the bioaccumulating characteristics of other organisms. In such a system, when the behavior of the triggering organisms indicates a detrimental change in water quality, water and tissue samples can be automatically collected. Subsequent analyses would determine abnormal levels in the water sample at the time the organisms was stressed, and a cumulative record of previous water content to assess chronic low-level exposure could be determined from analysis of the bioaccumulators.

Depending on the degree of automation, systems such as this could find almost immediate application in monitoring drinking water systems and waste water and storm sewer effluents. Indeed, as previously mentioned, alarm systems using fish behavioral responses are already in use in Europe.

- The panel agreed that additional refinement of chlorophyll measurement techniques would be only minimally fruitful. Although the ease with which measurements can be made lends itself to improvement, the overall fluorescence technique and equipment currently available is generally acceptable for most applications. The most important improvement desired is for an increase in data collection rate to keep up with the demand for the ground truth necessary for remote sensing verification, and not for additional detector development.

- Enzyme systems to detect and measure nitrate and nitrite have been developed, demonstrated, and await only the interest and attention of a private entrepreneur for reaching commercial production. Their particular attraction for immediate application would be in automated measurement systems for drinking water where sufficient markets should exist to make the necessary production engineering profitable.

- Enzyme systems for the near real time detection of toxic materials or classes of toxic compounds are currently under investigation and, while some systems have already been identified for limited application, others should be identified within the next few years. At the present time such systems can be used as real time alarms; however, their use as quantitative measurement devices holds some possibility with longer term development efforts.

- The phenomenon of bioluminescence also holds some promise as a gross indicator/alarm system for detection of toxic substances. Bioluminescence devices are being developed as replacement for LC50 laboratory tests and have not been evaluated for application as alarms. It is estimated however that such devices could have fairly low operational costs if used to automate manual systems. Although systems based on bioluminescence detectors would most likely be limited to detection levels higher than the more sensitive "fish alarm" systems, a cost analysis would reveal the areas of utility for such systems.

Long-Term Development

- Heterogeneous enzyme systems for detection and measurement of specific compounds offer development potential for the much longer term. These systems respond to a wide range of compounds of interest, are longer lasting, and can be made highly sensitive. While the development will involve considerable effort, potential benefit in terms of providing a net array of low cost sensing tools is also considerable. Development would benefit from close association with the medical instrumentation field.

- As long-term integrators, fish and mollusk blood chemistries were felt to offer considerable promise.

- Automated observation of plankton characteristics for sensing environmental change was felt to be impractical at present; however, a longer term development program could provide dramatic payoff in the future.

In addition to endorsing the continued investigation of biological concepts for use in development of environmental measurement systems, the panel made two specific recommendations for implementation within the Federal government.

(1) In areas where agencies have similar measurement responsibilities and interests, interagency technical reviews covering development

programs should be held. Although agencies may not necessarily be working on the same types of development, these reviews could stimulate interest and lead to greater awareness of new development possibilities and lead to possible cooperative efforts. To build the broadest support, these reviews should be held at the technical level.

(2) Any future Federal monitoring plan or plans should contain clear statements regarding specific measurement requirements. Without these statements, neither government nor industry can assess market requirements; consequently, it is unlikely that needed measurement and monitoring devices and systems will be developed and available when necessary.

Remarks
of
Ferris Webster, Assistant Administrator
for Research and Development
National Oceanic and Atmospheric Administration

at the

2nd Interagency Workshop on In-Situ Water
Quality Sensing: Biologicaal Sensors
Pensacola Beach, Florida

April 28, 1980

I am pleased to be with you today to deliver the keynote address at this workshop. To my knowledge, this workshop is the first of its kind. I know of no previous organized attempts to bring together experts in biology and engineering to combine ideas for new approaches to measuring pollution in water.

The subject of this workshop is important. Concern about pollution of the air we breathe and water we use is near the top of the list of problems with which our society must cope. Pollution is everywhere. Its nature is complex. We see the effects of pollution, but often do not understand the causes. We seek technological solutions to problems that are both technological and social.

Only a few years ago, the pollutants we were concerned with were limited to a few heavy metals and DDT. Today, because of our increased awareness, we are faced with a wide variety of toxic materials and hazardous substances. Both NOAA and the Environmental Protection Agency are concerned about the quantities of these wastes that have been disposed of in sensitive wetlands and coastal areas, and about the fact that our ability to detect and clean-up these areas is lacking.

Reliable information about the state of water quality is our primary need. This, in turn, calls for measurement systems and sensors. Understanding and controlling pollution, whether it be in air or water, requires observations of ecosystems and physical environments over long periods of time. In many cases, we must collect our measurements in environments alien to direct human observation. Innovation and technology transfer are needed. By technology transfer, I mean the application of new knowledge from one institutional setting to another. For example, space technology has provided us with the capability to remotely observe other planets. These successes have been transferred to other fields, many far removed from the space environment.

The examples of the application of new technologies in other fields can inspire us to seek such uses in the ocean. If we can now remmotely measure the composition of the surfaace of Mars, we should be able to examine the composition of the seafloor and riverbed sediments without laboriously taking grab samples. If we can now measure turbulence levels in blood vessels, perhaps we can do the same in the ocean to understand pollutant resuspension. In particular, advanced technologies in other fields might be used to improve our ability to observe ocean pollution and to understand its effects.

An obstacle to technology transfer often is the difficulty of communicating new knowledge in one field to another that might apply that new knowledge. Each field has its own jargon, its own journals, its own learned societies. Even in a single field, communications can be surprisingly difficult; across fields, they are generally accomplished only by determined people. Thus, this workshop is particularly important because it represents the first step in beginning a dialogue between disciplines that have traditionally been separated. Outside of the medical field, biologists and engineers have not often collaborated. In ocean pollution, we know that such collaboration is essential. This workshop, if successful, will add impetus to the development of the technology needed to understand and control ocean pollution.

The Federal Plan

Last December, 13 Federal agencies completed the first "Federal Plan for Ocean Pollution Research, Development, and Monitoring." This document was written in response to legislation passed by the Congress in May 1978. Under this law, NOAA is required to take the lead agency responsibility in preparing a plan for marine pollution studies. The plan must coordinate the programs of all Federal agencies involved in marine pollution studies. It must identify national needs and problems, establish priorities, describe existing Federal capabilities and their ability to meet the priorities, and recommend changes in the program. The recommended changes should respond to changes in goals, funding levels, pooling of efforts between Federal agencies, and elimination of duplication. I would like to mention some of the conclusions and recommendations of the plan that are particularly relevant to this workshop.

A major section of the Plan was entitled "Tools for Controlling and Minimizing Ocean Pollution." In that section, it was noted that the development of measurement technology affects all agencies and their programs, and furthermore, is frequently the limiting factor in developing information for dealing with ocean pollution problems. It went on to note that "state-of-the-art measurement of virtually all chemical and biological factors is limited to laboratory techniques as opposed to in-situ measurements," and that "methods are labor intensive, expensive, and not easily automated or transferred to the field." It continued to explain that "development in this area is frequently underemphasized because it often requires long lead times and cannot produce immediate results." Unfortunately, in the competition for scarce research dollars, we sometimes sacrifice research directed toward longer-term benefits. Consequently, we make few developmental breakthroughs in instrumentation and end up having the same chronic measurement problems year after year.

The plan recommends that special emphasis be given to the development of measurement technology that will benefit all agencies. It identifies the need for development of automated laboratory equipment, automated sampling equipment, and in-situ and portable measurement systems. All these recommendations are reflected in topics that you will be addressing in this workshop.

The Federal Plan focuses on marine pollution problems; however, we must keep in mind, that in the measurement area, marine and freshwater technologies have much in common. Many of the deficiencies that exist in the marine field are virtually the same as those in nonmarine areas. Consequently, improvements that result from the work you do here will benefit all the Federal agencies responsible for water pollution measurements and science in general.

NOAA's Marine Pollution Programs

I would like to take this opportunity, on EPA turf, to say a few words about NOAA's activities in marine pollution and, in particular, about our work in sensing technology. The Ocean Pollution Act calls for NOAA to establish a comprehensive, coordinated, and effective ocean pollution research and development and monitoring program. We have developed a broad marine pollution program throughout NOAA. Organizationally, in the Office of Research and Development, we have set up an Office of Marine Pollution Assessment, gathering together existing programs in environmental assessment, ocean dumping, and hazardous materials spill response. The Office of Fisheries has a special need for understanding the effects of pollution on marine habitats and has a strong program in the Northeast. In addition, we are developing plans to establish an operational marine pollution monitoring capability. The Environmental Data and Information Service is working on the special needs of marine pollution data management.

In technology development, NOAA is creating an Office of Ocean Technology and Engineering Services. This activity will be working with the Office of Marine Pollution Assessment to develop an expanded program of measurement technology. We must respond to increasing needs for technology in support of ocean pollution research and monitoring.

At the moment, we are addressing the development of selected sensors. A miniature dissolved-oxygen sensor has been developed which we hope will increase the reliability of dissolved oxygen measurement at a reduced cost. We are conducting research to determine whether aerospace image-processing, enhancement, and pattern-recognition technology can be applied to analyzing plankton samples. We are beginning research on the use of biodetection mechanisms such as enzymes and antibodies for selective sensing of toxic substances. A number of the researchers involved in these projects are here and will be describing this work.

NOAA is planning regional workshops and studies to determine optimum strategies for monitoring stressed areas throughout the U.S. coastal regionsal Our agency, along with others, represented here, has a commitment to the stewardship of the ocean environment. The increasing demand to watch over large ocean and coastal areas will require improved and innovative measurement tools that can sense trace quantities of toxic substances in the sediment, water column, and fish tissue. The sensors will have to do all these things and yet be housed in instruments that can be operated by low-skill-level personnel. We cannot afford to use research scientists to conduct routine surveillance. The results of this workshop can potentially benefit NOAA's pollution programs. We, and other agencies, need the kinds of capabilities you will be discussing here this week.

Summary

Biological organisms may hold the key to new breakthroughs in sensor concepts. Nature through evolutionary time has sharpened our senses and those of other living organisms with whom we share our planet. It is possible that sensitive and selective sensory mechanisms may be used directly to detect substances of interest. Perhaps even their mechanisms can be duplicated for use as indirect detectors in other applications.

I hope that during the next two days, ideas will be exchanged which will spark innovative approaches to sensor development. The four working panels that begin meeting tomorrow provide an opportunity to assess the state of biological research and technology and to recommend directions for future emphasis. I hope the personal interactions in this small meeting will stimulate and act as a catalyst to new thinking.

I am encouraged by the purpose of this workshop. I will await the results with interest. We in the Federal agencies can use your contributions in planning programs in this important and potentially fruitful area of biosensing.

BIOLOGICAL MONITORING OF HAZARDOUS WASTES IN AQUATIC SYSTEMS

by

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The Environmental Protection Agency (EPA) has initiated an aggressive program in the United States to discover, investigate, contain, and clean up uncontrolled (abandoned) hazardous waste sites that present an imminent hazard to human health or the environment. Under the authority of the Solid Waste Disposal Act, as amended by the Resource Conservation and Recovery Act of 1975 (and its amendments), the EPA is also responsible for the development, promulgation, and enforcement of regulations for the management and disposal of hazardous waste. These hazardous waste programs require measurement methods, monitoring and exposure assessment guidelines, field response, and a quality assurance program to provide accurate and reliable data for enforcement actions and for making proper management decisions to protect human health and the environment. One of the key elements and needs in the hazardous waste programs is biological monitoring. This paper provides an overview as to the needs for the application of biological monitoring in aquatic systems and its usefulness for monitoring hazardous wastes and their leachates.

NEED FOR BIOLOGICAL MONITORING

Biological monitoring is rapidly attaining the recognition it warrants as an invaluable component in comprehensive environmental monitoring programs. Indeed, for many years there has existed a bias toward the "hard numbers" generated by physical and chemical analysis and a reluctance to accept the intrinsically less precise values obtained with biological systems. The composition of monitoring staffs in the EPA and the predecessor agency, the Federal Water Pollution Control Administraation, has generally reflected the emphasis on physical and chemical measurement.

The reason for relegating biological monitoring to a weak supportive role in monitoring programs of the past were many. Some were valid. As viewed from the standpoint of the analytical chemist, the state-of-the-art of biological monitoring is still quite primitive. The water-quality biologist attempts to unravel the complexities of a highly intricate and variable system in an effort to determine if observed changes in the biota are real and, if so, whether these changes can be attributed to manmade influences.

It has been very difficult to effectively model many of the critical processes of biological systems. The biological responses of many test organisms are highly variable, even under rigidly controlled laboratory conditions, and responses determined under such conditions may or may not represent the responses of similar organisms in natural ecosystems. To effectively represent the environmental interactions of a single organism in a natural system is one of the greatest challenges to modelers and programmers today.

The use of major groups of organisms (e.g., aquatic diptera) as biological indicators has been hindered by lack of standardized and accepted classification schemes. While some significant efforts have been made to standardize taxonomies in recent years, much work remains to be done in the area.

Lack of biologically trained personnel has been more a consequence of inappropriate talent-mix selection - a product of the historical emphasis on physical/chemical measurement - than any lack of well-qualified biologists. A balance of disciplines which reflects today's monitoring needs should be achieved. In many cases this means the addition (or substitution) of biological personnel with training and experience appropriate to the monitoring program requirements.

Comprehensive biological data bases (e.g., The National Phytoplankton Data Base originally developed by EPA's National Eutrophication Survey) have been almost nonexistent. There has been limited opportunity, at best, to evaluate biological monitoring data against similar data obtained in the same manner by other groups. For example, the Chesapeake Bay Program, which has cost millions of dollars to execute, could have benefited greatly had the many years of excellent data - gathered by the collective organizations with research interests in the Bay - been available to all of the grantees/participants in some standard and uniform format.

The standardization of selected biological methods, from sample collection through data analysis, has done much to lend credibility to biological measurement techniques. However, biological monitoring is a dynamic and emerging field; new methods must be developed, tested, and evaluated and guidelines for their use issued. Biological measurement is being applied with great expectation in a new area, i.e., hazardous waste identification, for which it has not previously been evaluated. However, until biological data can consistently stand the tests of litigation, biological monitoring cannot come into its own as a primary regulatory tool.

The increasingly complex monitoring problems of today, as typified by hazardous waste sites monitoring, require sophisticated and broad-spectrum approaches for resolution. Accelerated development of methods, including biological methods, has been initiated to meet these needs. Many of the standards and controls promulgated by EPA are based upon biological testing and response. This is appropriate, as the primary mission of EPA is, simply stated, to safeguard human and environmental health; and often the first indications of impending problems are detected through impacts on the biota. Physical or chemical testing cannot predict the cumulative impacts of mixed pollution sources upon the biotic components of the environment. Synergistic or antagonistic effects can only be determined through biological measurements. Approaches that assess the "net" impact of "black-box" effluents or leachates upon biological community components will find increasing value as rapid screens or guides to subsequent analysis or control procedures.

The use of biological monitoring in conjunction with physical-chemical sensing is particularly advantageous in a systems approach. A strictly physical-chemical monitoring network that would monitor all suspected pollutants continually and throughout potentially impacted areas is very difficult and expensive to operate and could still fail to detect unsuspected or low-level pollutants. And often we don't have a complete list of pollutants which may impact water bodies.

However, analysis of biological samples, collected periodically from strategically located stations, could detect pollution-induced changes in the biota and provide an alert to hazardous conditions.

Foreign materials introduced into an aquatic environment interact in a complex and often nonlinear manner with one another and with the numerous other factors inherent to the environment. Aquatic organisms and communities respond to the sum of the interactions of these environmental factors. Thus, biomonitoring is particularly well suited to detecting changes in ambient conditions caused by both suspected and unsuspected foreign materials, even though the actual cause-effect relationships may be too complex to readily evaluate. Unfortunately, the often important effects of interactions between such factors as temperature, photoperiod, and flow rate, as well as toxic components in the ecosystem, can rarely be demonstrated in laboratory studies. In-situ biological monitoring remains the only effective way to measure the resulting complex impacts upon physiological and behavioral processes at the species, population, or community level.

An additional and very significant advantage of biological monitoring is that it provides a mechanism for the integration of conditions between sampling periods. Aquatic communities are affected by short-lived perturbations of the environment and these effects normally persist for the weeks or months required for the communities to recover. Biomonitoring can be used to detect short-term events that can have catastrophic effects and which chemical/physical monitoring is likely to miss. Biological monitoring is especially advantageous because it will detect the full spectrum of suspected and unsuspected impacts including manifestations of intermittent insults even through periodic sampling. Pollution is fundamentally a biological problem. We monitor certain chemical-physical parameters primarily because we know or suspect that they directly or indirectly impact living organisms.

Once biological monitoring has shown that there is a problem, intensive physical-chemical and biological monitoring could pinpoint both the "danger spots" and the causative agents and sources. As additional information becomes available concerning the relationships between specific pollutants and specific changes in the biota, the identificaiton of the pollutants and their sources will become easier. Only the complementary use of biological and physical-chemical monitoring will make it feasible to detect impacts and their sources over entire watershed systems.

Major legislation that establishes requirements for biological measurement, monitoring, and assessment include:

- Clean Water Act
- Toxic Substances Control Act (TOSCA)
- Solid Waste Disposal Act, as amended by the Resource Conservation and Recovery Act (RCRA)
- Federal Insecticide, Fungicide and Rodenticide Act (FIFRA)
- Safe Drinking Water Act
- Clean Air Act

Biological monitoring and assessment activities are presently conducted in support of such major efforts as:

- Evaluation of environmental impact statements
- Ecological assessments of spills or discharges of oil and hazardous materials
- Evaluation of the "Clean Lakes" restoration efforts
- Exposure assessment of toxic substances, pesticides
- Lake classification surveys
- Drinking water source evalutions
- Basin-wide planning
- NPDES compliance monitoring
- Pre- and post-construction evaluations of energy resource development

Areas in which it is anticipated that biological measurements will have increasing application include:

- Hazardous waste identification associated with active and uncontrolled waste disposal sites and industrial process streams
- Toxic substance and pesticide registration efforts
- Dredge spoil monitoring
- Ocean dumping impact assessment
- Acid-rain impact assessment
- Investigations relating exposure assessment to environmental and human health risks

APPROACHES TO BIOMONITORING

In-Situ Bioassays

Measurements of the toxicity of pollutants provide information on their impact upon living organisms. Usually, bioassays are conducted in the laboratory; however, they can be conducted in-situ in the field. The placement of caged animals, in an outfall or receiving stream, is a direct approach to the measurement of the impact of the discharge of hazardous substances to surface waters. In-situ bioassays are subject to vandalism, flooding, and a host of other uncontrollable factors including exposure of test organisms to unknown constituents and dosages for indefinite periods of time. While not impossible, it is difficult to determine long-term effects of pollutants in the field, as confinement of the test animals may alter their natural growth, life expectancy, and reproduction. The results of the in-situ bioassays are apparent when the caged organisms die; however, responses may be less pronounced, and often measurements of physiological or behavioral responses such as respiration rate, movement, reproductive success, and development of tumors are required to detect environmental effects. Chronic exposure to low-level concentrations may have no measurable effect on the test organisms themselves, but the effect of exposure may be manifested several generations later in the form of abnormalities resulting from such exposure.

Bioaccumulation

Living organisms have the ability to accumulate substances from the surrounding water medium and thus act as natural compositors and integrators of hazardous substances present in surface waters.

Plants and animals accumulate hazardous substances in tissue through bioconcentration (uptake from the water) and biomagnification (uptake through the food chain). For example, the concentration of radionuclides in algae at 10,000 times background levels have been documented, and the impacts of DDT upon top-of-the-food-chain organisms such as the osprey and brown pelican are well-known. The uptake characteristics of organisms vary by individual species and trophic level. Ideally, receptors at several trophic levels should be included in bioconcentration studies. The most obvious advantage of bioaccumulation studies is that they provide detection of hazardous materials, often at levels below analytical limits, and suggest the potential hazard to various food chain components, including man. Thus, these analyses offer an efficient means for screening and identifying potentially hazardous substances in water before they pose a serious risk to human or environmental health.

Indicator Organisms

Attempts have been made to use the abundance, presence, or absence of taxa (species or genera) as stand-alone indicators of environmental conditions. These assume that the pollutant or pollutants will have an effect on the indicator organisms and that it is possible to predict the abundance, presence, or absence of the organism in its unimpacted aquatic habitat. This is often very difficult, especially with groupings above the species level. For example, we have found that phytoplankton genera thrive over such a broad range of environmental conditions that they cannot be used as indicator organisms. In addition, seasonal variations in individual community components can effectively mask changes resulting from pollutant impact. Only with adequate base-line biological data can meaningful inferences be made under such conditions.

Community and Population Changes

The determination of community and population changes (e.g., species composition, diversity and abundance; standing crop; mortality rates; and community productivity) have been much more successful in measuring the response of aquatic communities to pollution-induced stress. However, it is difficult to obtain reliable measurements of this type because of the need to sample highly variable populations where composition and structure are subject to all the perturbations of the environment, both natural and man-induced. For example, immature aquatic insects are highly sensitive to pollution-induced stress conditions, but they also show large natural seasonal variations due to characteristics of their life cycles and natural fluctuations in stream conditions. In addition, identification of many groups of these insects poses special problems, due largely to the lack of taxonomic information on their immature stages. In spite of the diffficulties involved in characterization of aquatic communities, be they fish, invertebrates or plant life, carefully planned and executed investigations are well worth the effort. They offer a fairly efficient means of detecting pollution-induced stress once the natural community patterns have been fully described. Once again, however, the existence of "pre-impact" base-line data is invaluable in interpreting the nature and magnitude of the changes noted.

Species Health

The health of organisms can be used to determine if hazardous wastes are impacting the aquatic biota or are potentially harmful to man. Parameters which can be measured to determine species health include growth, reproduction, longevity, plumpness, tumor development, and phenotypes. Where possible, "control" measurements, e.g., from upstream-downstream studies or analogies with similar systems, should be obtained to determine the significance of the changes noted.

SELECTION OF BIOMONITORING TECHNIQUES

The factors listed below should be considered in selecting biomonitoring techniques.

- Status of development of the technique (e.g., testing and validation)
- Sensitivity (response to low concentrations of contaminants)
- Simplicity (how much training and experience required to conduct)
- Reproducibility (intra- and interlaboratory precision)
- Coverage (spectrum of contaminants to which technique is responsive)
- Cost (set-up, as well as sample-by-sample)
- Turnaround time (compatible with monitoring requirements)
- Confidence (past acceptance and success in application)
- Nature of response of the technique (relationship of response to human and/or environmental health)

Status of Development

While some of the biomonitoring techniques have been validated, many have not been evaluated and/or validated relative to specific program requirements, e.g., monitoring hazardous wastes. Some promising new techniques are still too developmental for serious consideration at this time although further evaluation and testing are strongly recommended.

Sensitivity

Particularly in characterizing complex waste mixtures, sensitivity to low concentrations of bioactive compounds is an important consideration in predicting the potential impact of that waste upon human and environmental health. Response to low concentrations of contaminants can often be enhanced through increasing the contact time of the test in the field. The time-sensitivity "trade-off" is only one of several that must be made in determining the final composition of selected biomonitoring techniques.

Simplicity

It is expected that personnel with a wide range of training and capabilities will be conducting and interpreting the biomonitoring. The more simple and straightforward the techniques are, the more likely comparable and meaningful results will be obtained by the spectrum of users. Even relatively straightforward procedures should be documented in sufficient detail to assure that data from various biomonitoring activities are comparable.

Reproducibility

Techniques which are less critical with respect to test conditions and yield similar results in the hands of a variety of operators are favored. In some cases, the "effective reproducibility" may be a function of the ambiguity of the test end point (e.g., subtle behavioral responses versus mortality), the clarity of test procedure documentation, or the skill of the operator in interpreting monitoring results. In general, only techniques that have been shown, through comparison studies, to give relatively uniform responses should be considered for general use.

Coverage

In assembling a battery of techniques to determine the impact on waterbodies, it is vital that there be as broad coverage of the spectrum of potential hazardous substances as possible. In general, "specialty tests" - for very limited groups of contaminants - have little general monitoring application unless the contaminants are of high priority and not effectively addressed by more broad-spectrum techniques. Synergistic or antagonistic effects of hazardous substances in the aquatic environment should be reflected in the biomonitoring results.

Cost

If the costs associated with individual techniques can be kept to a reasonable level, monitoring groups will be encouraged to use biological testing as part of their programs.

Turnaround Time

The time required to conduct many biological monitoring programs has hindered their general acceptance for many applications. Techniques requiring long-term baseline measurements (months or years) will not be used as extensively as those where meaningful interpretations can be made in a relatively short time frame.

Confidence

Somewhat less tangible, but nonetheless important, is the credibility gained by certain tried-and-true biomonitoring techniques. Often the confidence associated with performing "familiar" tests is reflected in the successful application of that technique. A sensitivity is developed to disturbances or interferences in the basic technique which often detects problems before they are reflected in bad data. Well established techniques also tend to carry the weight of precedence in enforcement proceedings.

Nature of Response

While the relationship of some biological tests to human health and welfare are rather clearly defined, other biological techniques bear more obscure relationships. Absence of direct pharmacological relevance does not rule out the consideration of specific techniques; however, strong empirical evidence is required to support such techniques.

CONCLUSIONS

Biological monitoring has had a relatively weak supportive role in monitoring programs of the past; however, it is rapidly attaining the recognition it warrants in a systems approach to monitoring. Physical and chemical testing cannot predict the cumulative impacts and/or the interactions of mixed pollution sources on environmental health. Biological monitoring is well-suited for this as the biota in aquatic systems responds to the sum of the interactions of environmental factors and integrates their effects over time. Biological monitoring is a dynamic and emerging field and new methods must be developed, tested, and evaluated to meet the challenging monitoring requirements associated with the identification and assessment of hazardous wastes.

For selection of appropriate biomonitoring techniques,

- The monitoring requirements of a specific program should be clearly determined,
- The selection criteria provided should be reviewed and prioritized relative to those monitoring requirements identified, and
- Finally, the selection criteria should be rigorously applied in determining those tests or measurements needed to meet program objectives

Biological monitoring methods which are sensitive, simple to apply, reproducible, and economical in their application, and which provide answers to vital environmental questions in realistic time frames, represent a goal toward which our research and monitoring groups must continually strive.

ENZYMES AND OTHER BIOCHEMICAL INDICATORS OF TOXICANT EFFECTS IN FISHES[1]

by

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The major purpose of this workshop is to evaluate in-situ biological sensors of water quality. This paper will provide an overview of the subject of enzymes and other biochemical indicators of toxicant effects in aquatic organisms, especially fish. To protect endemic and economically important fish within a particular ecosystem, water-quality biologists must measure biochemical indicators in fish species that have sensitivities comparable to the fish in the ecosystem. Because I represent the Great Lakes Fishery Laboratory, I will emphasize the Laurentian Great Lakes, an ecosystem containing valuable fishery resources.

The Great Lakes

The Laurentian Great Lakes, having 95,000 square miles of water surface and extending 2,000 miles, are the world's largest reservoir of fresh water. For more than a century, many of the largest and most successful cities and industries of the Midwest have depended on this water as a vital element in their growth. Billions of gallons of municipal, industrial, and agricultural wastes have been discharged into these lakes because of the convenience of doing so. The dumping of wastes and physical alteration of the shores and waterways has been at the expense of the aquatic ecosystem. The fish communities of each of the Great Lakes have undergone marked transformation, especially during the last half century. Populations of endemic climax fish like the lake trout (*Salvelinus namaycush*), walleye (*Stizostedion vitreum*), and coregonid species have been drastically reduced as a result of overexploitation; invasion of the Great Lakes by sea lampreys (*Petromyzon marinus*), alewives (*Alosa pseudoharengus*), and rainbow smelt (*Osmerus mordax*); introduction of Pacific salmon (*Oncorhynchus* spp.); and degraded environmental quality.

A problem of major concern in Great Lakes research is the apparent reproductive failure of lake trout in the Great Lakes. Lake trout spawn and produce viable gametes, but no survival past the fry stage has been observed except in Lake Superior. Although lake trout once supported valuable commercial fisheries in each of the Great Lakes, the species was probably extinct by 1956 in all but Lake Superior, which retained a residual population. The decline and near extinction of the species are believed to have been caused by a combination of excessive exploitation and of heavy predation by the sea lamprey, which invaded the Great Lakes through man-made canals connecting the lakes with the St. Lawrence River and the sea. In spite of control of the sea lamprey and the planting of millions of lake trout in the upper three lakes (Superior, Michigan,

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Huron) since the mid-1960s, natural reproduction of planted fish has been known to occur in only two locations. Young fry have been collected in grand Traverse Bay (Lake Michigan) and Marquette harbor (Lake Superior). After a decade of research, the roles of various biological and environmental factors, including contaminants, are not yet clearly understood.

Over the past 10 years, several major contaminants, notably mercury, DDT, PCBs, dieldrin, and mirex, have posed a threat to the Great Lakes ecosystem, caused curtailments of commercial fishing, and resulted in warnings to sport fishermen against consumption of fish. Regulation of the sources has resulted in a general decline of most of these contaminants, although substantial residues remain in the sediments and aquatic biota.

New contaminants continue to be identified in the Great Lakes; among the more recent are chlorinated benzenes, chlorinated styrenes, toxaphene-like compounds, and dioxin. Aquatic toxicological data are needed for use in hazard assessment schemes that are being developed to avoid future crisis situations like those with mercury, DDT, and PCBs.

Great Lakes Fishery Laboratory

The Great Lakes Fishery Laboratory, Ann Arbor, Michigan, is administered by the Division of Fishery Ecology Research of the U.S. Fish and Wildlife Service. The mission of the Laboratory is to develop the knowledge and the technical basis for assessing, protecting, enhancing, and rehabilitating the valuable fishery resources of the Great Lakes and their habitat. This mission is pursued by a staff of over a hundred. The Laboratory operates five major research vessels, which are used to survey Great Lakes fish stocks with trawls, gillnets, larval-fish tow nets, hydroacoustic fish-detection systems, and other gears, and sample their habitat for information about its limnological characteristics, including the presence of toxic substances.

The Laboratory, together with State and Canadian resource agencies, is working to determine the distribution and abundance of important sport and food fishes, the effects of fishing and other mortality agents on the stocks of these fishes, and the size and potential of populations of forage fish like the alewife and smelt, on which important game and food fishes feed. The Laboratory also tries to project the effects on fishery resources of alterations of the habitats caused, by example, by pollution, water withdrawal for power-plant cooling systems, channel-maintenance dredging, and shoreline modification such as bulkheading and draining marshes.

Contaminant research at the Great Lakes Fisher Laboratory, which is funded by the Service's Environmental Contaminants Evaluation (ECE) program, is divided into two projects - Contaminant Toxicology, and Contaminant Dynamics - comprising eight work units. (See appendix A.) (Other ECE research units outside this Laboratory are listed in appendixes B and C.) The Contaminant Dynamics project is involved in studies of contaminant uptake from water and sediments, trend monitoring, and, more recently, field studies on effects of contaminants. The Contaminant Toxicology project, of which I am the leader, was initially oriented toward mode-of-action studies of contaminant effects on enzymes. Although the emphasis of the project has changed toward whole-animal studies, my associates and I will in the future measure enzymes and other biochemicals in aquatic organisms whenever this approach seems likely to provide useful information to managers of fishery resource and water quality.

Enzymes

General review. For about two decades, water quality biologists have been searching for sensitive indicators of sublethal effects of contaminants to attempt to understand the mode of action of toxicants, and to develop a basis for corrective action in cleaning up water bodies before the health of aquatic populations is seriously threatened. Enzymes are attractive as indicators because they are more easily quantified than are other indicators of sublethal contaminant effects, such as changes in behavior. Useful precedents have been set in clinical medicine in the successful diagnosis of disease and evaluation of exposure to industrial chemicals or drugs by analyses of such variables as enzymes, blood chemistry, and liver function. In the fishery field, however, enzyme research is in its infancy: only two fish enzymes have yet been proven to be useful indicators of toxic effects of specific contaminants or classes of contaminants.

The first of these enzymes is acetylcholinesterase, which modulates the amounts of the neurotransmitter acetylcholine in the nervous system. The mode of action of malathion, parathion, and other widely used organophosphate insecticides is inhibition of this enzyme. Measurement of acetylcholinesterase in the brains of distressed fish collected in the field has been successfully used to diagnose organophosphate poisoning of fish in natural waters (Coppage et al., 1975).

The second indicator enzyme is delta-amino levulinic acid dehydratase (ALA-D), which is in the pathway of heme synthesis. Fish exposed chronically to lead suffer from anemia, which is probably a result of the disturbance of hemoglobin synthesis by the blocking effect of lead on ALA-D. Inasmuch as Hodson et al. (1977) reported that near-lethal exposures of rainbow trout (*Salmo gairdneri*) to cadmium, copper, zinc, and mercury did not significantly inhibit erythrocyte ALA-D, the enzyme appears to be specific for lead. In addition, the concentrations of lead that inhibit ALA-D activity during short-term exposures of fish are similar to concentrations causing other sublethal symptoms such as black tail, spinal curvature, and erosion of the caudal area of rainbow trout after many months of exposure. Hence, ALA-D fits the criteria for a short-term enzyme indicator of long-term sublethal effects of a contaminant on fish. The Columbia National Fishery Research Laboratory of the Service will be measuring blood ALA-D in fish exposed environmentally to lead released from mine tailings in Missouri. Their results may provide field verification of the usefulness of this enzyme.

An enzyme that shows promise as an indicator is adenosine triphosphatase (ATPase), a widely distributed enzyme involved in the energy-requiring active transport of electrolytes across membranes. The pesticide DDT inhibits mitochondrial ATPase from several fish tissues. However, different types of ATPases from some tissues may be activated in response to toxicants. The ATPases are not specific indicators of DDT exposure - inhibition by toxaphene, PCBs, and pyrethrins has also been reported.

Another approach to using enzymes as indicators of toxicant exposure is measuring the enzymes that metabolize or detoxify contaminants. Generally, when animals are exposed to toxicants, the detoxifying enzymes are induced - that is, more enzyme is synthesized.

A good example of how detoxifying enzymes can be used to monitor specific pollutants was given by Walton et al. (1978), who found that hepatic aryl hydrocarbon hydroxylase (AHH) in the cunner (Tautogolabrus adspersus), a marine fish living off New England, was a sensitive indicator of petroleum contamination. Oil concentrations of 1 to 2 mg/l caused twofold to sixfold induction of AHH above normal levels. Observation of elevated AHH followed oil contamination by 1 or 2 days. Because induction of AHH decayed in less than 7 days after exposure ceased, it reflected the current state of oil contamination. Feeding of crude oil or the tissue of mussels contaminated with oil at a concentration of 500 mg/kg caused up to fivefold induction. In-vitro tests with trout showed that AHH was not induced by PCBs, or by representative organochlorine, organophosphate, or carbamate pesticides.

Research at Great Lakes Fishery Laboratory. We did some in-situ enzyme work in the Contaminant Toxicology project a few years ago. After completing in-vitro experiments to determine effects of heavy metals, PCBs, and DDT on the uricolytic enzyme allantoinase in lake trout liver (Passino and Cotant, 1979) and in-vivo experiments with PCBs, we collected lake trout from two areas - one near Isle Royale, a clean area in northern Lake Superior, and one near Saugatuck, Michigan, a moderately contaminated area influenced by industrialization, typical of southeastern Lake Michigan. We found significantly ($P < 0.01$) greater enzyme activity in small than in large lake trout from Lake Michigan. (See table 1.) Enzyme activity was also higher in the smaller fish from Lake Superior and from stocks in the Laboratory, but not significantly so.

Although the interpretation of data from field samples is complicated by many unknown variables operating on enzyme systems in fish, we demonstrated an inverse correlation between enzyme activity and length of lake trout. This clear trend in activity may have been caused in part either by the greater age of the longer fish, or by higher levels of contaminants in older fish - especially in Lake Michigan. (See table 2.) This example illustrates the difficulty in establishing a causal relationship between residue levels and observed enzyme activities in fish collected in the field. The only exceptions may arise after clear-cut demonstration in the laboratory of specificity of a particular contaminant or class of contaminants for a particular enzyme.

Another piece of work related to enzymes was that of measuring the subcellular distribution of chemical contaminants in lake trout liver. Such determination holds promise as a means for predicting which metabolic pathways are most likely to be affected, because each subcellular fraction or organelle is specialized for particular functions. Although we investigated mercury, PCBs, and DDT plus DDE, only the data on mercury are relatively free of problems. These data were obtained by collecting large live lake trout in Lake Michigan near Saugatuck, bringing it live to the laboratory, killing it, and immediately removing the liver and separating the subcellular fractions by differential centrifugation. One set of subcellular fractions was examined by electron microscopy to quantify the organelles present and the other set was analyzed for total mercury by a combustion amalgamation technique. (In addition, we had earlier obtained data from a frozen liver.) The organelles of interest are shown in figure 1. Mercury was primarily (80%) in the submicroscopic material (see figure 2) - that is, in material so small it could not be seen at 7,700 x magnification. Of the remainder, 5% was in the mitochondria, 5% in the rough endosplasmic reticulum, and 1 to 2% in some of the other organelles.

What does this distribution mean in terms of fish metabolism and the health of the fish? In other vertebrates, such as the rat, a metal-binding protein, metallothionein, has been found in liver and kidney. This protein serves as a protective mechanism in animals, because the protein-bound metal is not available to interfere with metabolic processes. Therefore, I suggest that lake trout may be able to protect themselves to a large extent from the toxic effects of mercury by concentrating mercury in the submicroscopic material of the liver, where the mercury is probably bound to a metallothionein-like protein. However, since other recent investigations on rainbow trout and goldfish (*Carassius auratus*) (Olson et al., 1978; Marafante, 1976) showed that no more than 50% of mercury was bound to metallothionein-like proteins, some interference of mercury with metabolic pathways in the cytosol is possible. These pathways include fatty acid synthesis, glycolysis, many reactions in gluconeogenesis, the hexose monophosphate shunt, and activation of amino acids for protein synthesis.

Similar results were obtained for the distribution of PCBs and DDT plus DDE in frozen liver. Although 96 to 97% of these organochlorines were found in the soluble fraction, the mitochondria were ruptured during the freezing and thawing process; consequently, confirming work needs to be done with fresh liver. The logical follow-up research would be to determine if the levels of contaminants present are inhibitory to key enzymes in these fractions, especially those in the cytosol. We did not do the follow-up research because it would have entailed a mode-of-action study at the subcellular level. Because fishery and water quality managers need to have technical information that is directly useful in their decision-making process, we are now conducting whole-animal studies, with emphasis on effects on growth and reproduction. Changes in biochemical indicators in animals exposed to toxicants can be useful in predicting long-term effects on populations.

Other Biochemical Indicators

As part of the continuing research on the reproductive failure of lake trout in Lake Michigan, we have been involved in two interproject studies (with the Contaminant Dynamics project) to evaluate the toxicity of major organochlorine contaminants to lake trout eggs and fry, which are critical life stages. In one study (1977), 20,000 fry from Lake Michigan lake trout were exposed to simulated Lake Michigan levels of PCBs (Aroclor 1254) and DDE, and to levels 5 and 25 x higher, in food and water. Ambient levels were 10 ng/l PCBs and 1.0 ng/l DDE in water and 1.0 μg/g PCBs and 0.1 μg/g DDE in food. Cumulative mortality of the fry exposed to simulated Lake Michigan levels of PCBs and DDE for 6 months was 40.7 - nearly twice that of unexposed (control) fry - and mortality at the highest exposure level was 46.5%. We concluded that, although several factors have undoubtedly contributed to the reproductive failure of lake trout, the levels of PCBs and DDE in Lake Michigan during the early and mid-1970s were high enough to significantly reduce survival of fry in the lake and thereby impede restoration of the lake trout population to self-sustainability.

During the course of this study, two interesting trends were observed that warrant further investigation. One trend was toward a slight increase in growth rate of the exposed fry. A second trend was toward an alteration in glycolysis in exposed fry that had been exercised to exhaustion in a swimming apparatus. Further work needs to be done to measure oxygen consumption, muscle lactate, and muscle glycogen in fry before and after swimming to exhaustion to determine if their stamina is affected by exposure to organochlorine contaminants.

In the second interproject study (1979), we exposed eggs and fry from Lake Michigan lake trout to simulated Lake Michigan levels of organochlorines. Nominal concentrations were 1 ng/l PCBs (Aroclor 1254), 0.2 ng/l DDE, 1 ng/l dieldrin, and 0.9 ng/l technical grade chlordane, and 10 x multiples of these concentrations. No differences in mortality were observed between exposed and unexposed eggs and fry through 180 days of exposure, to the "swim-up" stage. Measurements of lengths taken from the time of hatch almost until swim-up (age about 60 days) showed a trend, significant at the 10% level, for increased growth of fry exposed to the lower levels of organochlorines, by time of swim-up.

We also used a biochemical indicator of instantaneous growth by measuring RNA-DNA ratios. A high RNA-DNA ratio is correlated with rapid protein synthesis and weight gain. At swim-up, the RNA-DNA-ratio of the control fry (3.85 ± 0.11 (± SE) was significantly lower ($P < 0.10$) than that of the fry receiving higher levels of the organochlorines (4.38 ± 0.04). The RNA-DNA ratio for the low treatment fry was intermediate (4.08 ± 0.15). Thus the trend toward increased growth of fry exposed to organochlorines was supported by the RNA-DNA data.

Kearns and Atchison (1979) demonstrated that field-collected yellow perch (*Perca flavescens*) showing different growth rates, as measured by changes in weight or length, had parallel changes in the RNA-DNA ratios. In their study, the growth rate of yellow perch exposed environmentally to cadmium and zinc from an electroplating plant was slower than that of controls in a cleaner area upstream from the plant. However, contaminated fish grew fastest in late summer, and uncontaminated fish in midsummer. The authors cautioned against comparing growth rates of fish in two populations at a single sampling period. Measurement of RNA-DNA ratios shows promise as an in-situ indicator of contaminant effects.

Other biochemical indicators that could be examined when investigating reproductive failure of fish populations are reproductive hormones. The importance of reproductive hormones in enabling fish to spawn and produce normal young is well known. Hence, alterations in reproductive hormone levels by exposure to contaminants is a serious threat to the productivity of populations. Sangalang and Freeman (1974) monitored testosterone and 11-ketotestosterone in brook trout (*Salvelinus fontinalis*) during low-level (1 μg/l) exposure to cadmium. The concentrations of these two androgens were different in the fish exposed to cadmium, suggesting impairment in the clearance and use of testosterone and 11-ketotestosterone. The impaired clearance appeared to be related to the abnormal vasculature and hemorrhagic necrosis observed in the testes of some of the cadmium-treated fish. These results are significant because brook trout spawn in rivers and streams that are prone to industrial pollution. Further studies are needed of fish reproductive hormones, especially field verification by the measurement of reproductive hormone levels in environmentally exposed fish.

Investigators at the Columbia National Fishery Research Laboratory, who used bone composition and strength as sublethal indicators of contaminant effects, found that toxaphene exposure of fathead minnows led to decreased collagen concentration, altered amino acid composition, increased calcium concentration, and decreased bone strength of the vertebrae (Mehrle and Mayer, 1975). These biochemical manifestations of the "broken back" syndrome have been recently observed in striped bass (Morone saxatilis) collected in tributaries of Chesapeake Bay. Striped bass with higher body burdens of organochlorines and metals have lower collagen and decreased bone strength.

Other types of biochemical indicators are those associated with the response of fish to stress, contaminants being one type of stress. Response to stress involves both the primary effects (hormones) and secondary effects, as shown in figure 3. Steroid hormones that have been investigated relative to contaminant effects are corticosteroid (cortisol, corticosterone) and catecholamines (epinephrine, norepinephrine). These hormones are part of the adrenal-pituitary response of vertebrates to stress. In our earlier (1977) study on lake trout fry, we measured cortisol by radio immunoassay. No differences were found between exposed and unexposed fry. However, the variability of cortisol within treatments was high. The fry were too small for sampling blood and the cortisol had to be extracted with methylene chloride from homogenized whole fry. Although cortisol may have been elevated during the initial exposure of the fry, the fish may have become adapted to the low-level chronic exposure.

Schreck and Lorz (1978) found that exposure to copper resulted in a marked, dose-dependent serum cortisol elevation in coho salmon (Oncorhynchus kisutch). However, treatment with cadmium did not elicit a cortisol elevation, even in moribund fish. They speculated that some but not all stresses produce elevations of cortisol, and that the mammalian concept of General Adaptation Syndrome may not completely apply to fish. In addition, cortisol may not be useful to detect effects of continuous exposure to sublethal contaminants, since cortisol is elevated primarily during the initial period of exposure to the stressful agent and may later return to normal levels.

Secondary response to stress includes changes in blood glucose and lactate, plasma free fatty acids, electrolyte balance, liver glycogen, immunosuppression, etc. (Mazeaud et al., 1977). The clinical approach to blood chemistry is successfully applied to human beings because normal baseline values for such properties as electrolytes, glucose, and hematocrit have been established. For fish, however, such baseline values have yet to be established, especially for wild populations, although some progress is being made. Since homeostasis is not so finely tuned in poikilothermous fish as in homothermous animals, the range for normal values is much broader for fish. Consequently, deviations from normal must be greater to be statistically significant. In addition, the physiology of fish is profoundly influenced by factors such as season, spawning condition, and migrations.

Research and Instrument Needs

Research. The above statements point out some of the challenges in applying biochemical information in a way that is helpful to managers of fishery resources and water quality, especially those charged with establishing water-quality criteria. An observed change in a biochemical indicator is significant to a manager only if it can be shown to influence higher levels of organization, ultimately the population level. An observed effect must be recognizable as being caused by contaminants and not by natural or endogenous agents. Background data on normal values and expected variability are required. The biochemical indicator to be considered for field monitoring should not be altered by the stress caused in capturing the fish. Because of these and other requirements, blood chemistry indicators such as glucose, electrolytes, and hemoglobin are not now particularly very useful because of the difficulties of establishing normal values and the changes that occur during capture of the fish. Enzymes that are specific for particular contaminants appear to be the most promising, together with reproductive hormones that show a significant response to contaminants. More research is needed to find enzymes and hormones that show specificity toward particular contaminants. Most likely, no one biochemical indicator will suffice to diagnose the health of fish, but rather a combination of indicators. Practitioners of clinical medicine normally measure an array of physiological and biochemical indicators before diagnosing disease. Wedemeyer and Yasutake (1977) have published methods for clinical indicators useful in assessing the health of fish populations.

Research is needed to integrate laboratory and field studies and to develop correlations between adverse effects in laboratory animals and adverse effects in wild populations. Laboratory studies provide high precision for predicting effects of individual contaminants under controlled conditions, but these studies fail to duplicate the many interacting environmental variables. Field studies address real environmental problems but fail to establish cause-and-effect relationships and are usually not sensitive enough to detect adverse effects before they reach crisis proportions. Although the emphasis of this workshop is on in-situ or field indicators, it must be remembered that an integrated approach with both field and laboratory studies is needed to interpret and understand the response of aquatic organisms to contaminants.

Instruments. Although this paper is primarily an overview, a few equipment or chemical needs should be mentioned. First, much time could be saved by biologists if they did not need to construct their own diluters for bioassays. Ideally, such marketed diluters should be portable enough to fit into a mobile or floating laboratory. Second, in-situ analytical monitors of chemical concentrations are needed in bioassay systems. These monitors could be used either for the toxicant in the bioassay system or perhaps for serum enzymes in larger fish. Some automated enzyme systems are already available for clinical laboratories and might be adapted for use with fish. Monitors for toxicants in a bioassay system would not need to identify unknowns, but only quantify a known chemical. Lastly, some fish hormones look promising as indicators of contaminant effects. The most sensitive methods generally are radioimmunoassay. However, all the available kits for radioimmunoassay are based on human antigens. Marketing of kits with antibodies to fish antigens is needed. Other specific instrument or chemical needs are considered in other papers in this compilation.

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APPENDIX A. ENVIRONMENTAL CONTAMINANTS EVALUATION - CURRENT AND FUTURE WORK UNITS AT THE GREAT LAKES FISHERY LABORATORY

Contaminant Toxicology Project

1. Effects of contaminants on the invertebrate forage base of Great Lakes fish populations.
2. Effects of toxic substances on metabolism of fry from Lake Michigan lake trout.
3. Toxicological evaluation of recently identified contaminants in the Great Lakes.

Contaminant Dynamics Project

1. Surveillance of contaminants in Great Lakes fishes.
2. Effects of multiple-contaminant exposures of eggs and fry of Lake Michigan lake trout.
3. Seasonal variation of organic contaminants and lipids in Lake Michigan fishes.
4. Size-specific mortality of Great Lakes lake trout fry with different burdens of contaminants.
5. Survival of lake trout eggs and fry in waters of the Upper Great Lakes.

APPENDIX B. ENVIRONMENTAL CONTAMINANTS EVALUATION - CURRENT WORK UNITS AT THE COLUMBIA NATIONAL FISHERY RESEARCH LABORATORY

Columbia National Fishery Research Laboratory, MO.

1. Acute toxicity of environmental contaminants to freshwater fish and fish food organisms.
2. Development of methods and experimental conditions for assessing the acute toxicity of toxic materials and contaminants, identified in monitoring programs, to fish and fish food organisms.
3. Influence of temperature, turbidity, and pH on the toxicity of insecticides and herbicides used on cotton and soybeans to fish and fish food organisms.
4. Susceptibility of fish and fish food organisms following sublethal exposures to major classes of environmental contaminants.
5. Analysis of the relationship between physical, chemical, and biological properties of toxic chemicals for predictions of biological effects in fishery resources.
6. Influence of natural environmental factors on chronic toxicity and residue dynamics of hexachlorocyclopentadiene in the fathead minnow.
7. Impact of inorganic contaminants from mineral and energy development on aquatic plants and salmonid fisheries of the Western U.S.
8. Integration and correlation of toxicological parameters for comprehensive hazard assessment of PCB replacements and herbicides.
9. Biological significance of aquatic contaminant residues identified by the National Trend Monitoring Program (e.g., phenolics and industrial intermediates) to fish food organisms, salmonids, and channel catfish.
10. Effects of herbicides used in hardwood forest and range management practices on the reproduction of daphnids and emergence of midges.
11. Development of methods and experimental conditions for chronic toxicity studies to better evaluate the impact of toxic chemicals on fishery resources.
12. Collation and assessment of information, and formulation of research design and objectives, to investigate suspected and future contaminants of critical aquatic resources.
13. Partial-chronic studies comparing intermittent and continuous exposures of rainbow trout to toxic pollutants.

14. Development of laboratory procedures for assessing the influence of contaminants on rates of detritus conversion in streams impacted by forest management practices and land-use changes.

15. The secondary impact of agricultural chemicals (dimilin, dylox, sevin) on warmwater fish production due to destruction of invertebrate forage.

16. Investigations of the effect of pentachlorophenol (herbicide, wood preservative) on the productivity of warmwater ecosystems, with emphasis on the limnological factors that contribute most to ecosystem stability.

17. The fate of pentachlorophenol (herbicide, wood preservative) in warmwater ecosystems: uptake, persistence, degradation, and effects on aquatic plants and fish food organisms.

18. Effects of herbicides used in and along irrigation and waterway canals on survival and development of rainbow trout eggs and fry.

19. Feasibility of "herding" fish with copper sulfate and other selected chemicals out of ponds and irrigation canals to be treated with toxic herbicides.

20. Effects of controlled-release formulations of aquatic herbicides used in irrigation canals on survival of rainbow trout and invertebrates.

21. Field evaluation of chemically "herding" fish under canal and reservoir conditions.

22. Delayed toxic effects of sublethal exposure of salmonids to aquatic herbicides associated with irrigation systems.

23. Salinity tolerance of hybrid grass carp.

24. Toxicity and biological availability of contaminants associated with particulate matter from coal-fired power plants to yellow perch and fish food organisms.

25. Effects of drilling and of crude and refined petroleum products on walleye, yellow perch, and aquatic food organisms of the Northern Great Plains.

26. Impact of herbicides associated with no-till agriculture on the fishery of the Northern Great Plains.

27. Toxicologic screening of contaminants against naiads of stoneflies and fingerling cutthroat trout.

28. Avoidance of selected herbicides and Wyoming crude oil by cutthroat trout.

29. Toxicity of mixtures of range management chemicals to cutthroat trout.

30. Accumulation in cutthroat trout of different hydrocarbon fractions from a reconsituted crude oil and the resulting biological effects.

31. Accumulation in cutthroat trout and resulting biological effects from petroleum hydrocarbons lost to surface water during refinement of crude oil.

32. Effects of several candidate mosquito insecticides on survival, drift, and emergence of *Pteronarcella badia* under simulated use patterns of exposure.

33. Development of hazard assessment techniques for evaluating the sublethal impact of toxic chemicals on reproduction, growth, and adaptability in rainbow trout, brook trout, and channel catfish.

34. Application of biochemical, physiological, and histopathological techniques to field monitoring studies: development of biological indicator techniques for assessing the impact of agricultural, industrial, and energy-related chemicals on fishery resources.

35. Influence of industrial and agricultural chemicals on biochemical profiles of mixed function oxidases (MFO) and related enzymes in fish and invertebrates from the Great Lakes and Lower Mississippi Valley.

36. Impact of contaminants from mining and energy development on critical habitat for Northwestern and Alaskan salmonid and anadromous fish populations.

37. Microbial degration of toxic industrial chemicals in freshwater hydrosoil of streams, lakes, and reservoirs.

38. Development of laboratory techniques to study the effects of toxic industrial and agricultural chemical contamination on microbial geographical cycle in streams, lakes, and reservoirs.

39. Disturbance of aquatic geochemical cycles by inorganic contaminants resulting from energy development in western United States.

40. Identification and determination of the impact in fish of metabolic products or organophosphate esters, replacements for PCBs used in hydraulic fluids.

41. Interpret the identity and impact of phenolic degradation products stemming from wood preservatives and industrial processes in eastern and southeastern rivers: laboratory metabolism and toxicity studies on important lower Mississippi River fishes.

42. Methods development and validation required to analyze samples of chronically and acutely exposed fathead minnows, brook trout and fish food organisms: emphasis on phenols (drilling fluids and wood preservatives), organophosphate insecticides, and industrial chemicals.

43. Discovery of exotic and other unknown contaminants in biota from critical habitats and from NPMP investigations: methods for fractionation of heterogenous chemical complexes such as phenols, polynuclear aromatic hydrocarbons, and heterocyclic compounds in water, sediments, and fish.

44. Development of analytical methodology for measurement (in water, fish, and fish food organisms) of heterocyclic and other herbicides used in intensive agricultural practices.

45. Methodology survey and development for analysis and assessment of polynuclear aromatics and other toxic compounds in water, sediment, and fish in intermountain streams, related to energy development.

46. Development and application of innovative materials for separating trace organics isolated from aquatic organisms: emphasis on herbicides, phenols, and polynuclear aromatics.

47. Analytical residue support for laboratory toxicity studies designed to evaluate the impact of environmental contaminants, including pentachlorophenol (wood preservative), phosphate esters (PCB substitutes), phthalate esters (industrial plasticizers), organophosphate insecticides, and carbamate pesticides, on aquatic resources.

48. Analytical residue support for agencies involved in the assessment and mitigation of pollution and fish and wildlife kills.

49. Trends in levels of contaminants in fisheries of the Nation's major lakes and streams: National Pesticide Monitoring Program.

50. Effects of samples handing and storage conditions on NPMP samples: Shelf life of selected inorganic and organic contaminant residues in fish.

51. Sampling and analysis of fish tissues for toxic substances.

52. The ecological effects of contaminants such as PCB replacements on stream communities as determined under controlled conditions in artificial streams.

53. Disturbance of aquatic geochemical cycles by PCB and PCB replacements in streams, lakes, and reservoirs.

54. Chronic toxicity of PCB and PCB replacements on fish and fish food organisms in the Upper Mississippi River.

55. Effect of PCB replacements on the behavior of Northwestern and Alaskan salmonids and anadromous fish.

56. Impact of PCB and PCB replacements on aquatic snails (e.g., Campeloma sp.).

57. Identification and assessment of metabolic degradation products of PCBs in Great Lake salmonids and panfish of the Upper Mississippi River.

58. Development of analytical methodology for toxicity evaluation of PCB substitutes: emphasis on organophosphate and phthalate esters and chlorinated benzene dielectrics.

59. Determine accumulation and distribution of structurally related *ortho*, *ortho*'-chlorine PCBs in biota and other aquatic compartments of the Upper Mississippi River and organisms from related laboratory studies for model development.

60. Identification and analysis of chlorinated dibenzofurans (highly toxic contaminants present in PCBs and chlorinated phenols) in fish collected from NPMP and field survey investigations.

61. Dynamic transport and deposition of polychlorinated biphenyls in fish and waterfowl habitat of the Upper Mississippi River.

62. Relative efficiency of important fish and diving duck food organisms to bioaccumulate PCBs, and their biological effects.

63. Biological availability of PCBs associated with the habitat of prime food organisms of fish and waterfowl of the Upper Mississippi River.

64. Physical-chemical aspects of bioconcentration, including aqueous partitioning, that affect the availability of PCBs.

65 . Analytical support for research in the Nation's watersheds to study impact of acid-metal precipitation, changing land-use practices, irrigation return waters, and energy development on aquatic resources.

66. Preliminary studies to evaluate amino acid levels and incidence of neoplasms as indicators of contaminant stress on aquatic biota.

67. Identification of detected but unknown toxic organic contamnants in biota from NPMP.

68. Collation and assessment of field information and formulation of research design to investigate suspected and future contaminants on critical aquatic resources.

69. Evaluation of occurrence of bilateral asymmetry in yellow perch as a new method for indicating aquatic contamination.

70. Sublethal effects of Sevin-4 oil and othe forest insecticides on natural populations of salmonids in Maine streams.

71. Impact of acid rain and metals from stack and auto emissions on brook trout, Atlantic salmon, and other fishes of the Northeast.

72. Impact of increased agricultural chemical usage on aquatic environments following the removal of bottomland hardwood forests in the lower Mississippi Alluvial Rivers.

73. Effects of contaminated irrigation return flows on striped bass and other anadromous and resident fish popultions of the Lower San Joaquin and Sacramento rivers.

74. Effects of brine discharges from petroleum wells on faunal composition of fish and fish food organisms in Oklahoma streams.

75. Effects of forest management herbicides on anadromous salmonids and fish food organisms in streams of the Pacific Northwest.

76. Effects of removal of riparian vegetation and increased sediment load on coho salmon and other anadromous salmonid populations in the Pacific Northwest.

77. The role of suspended sediment in determining bioavailability, toxicity, and longevity of some important agricultural herbicides in aquatic environments of the Missouri River System.

78. Effects of copper-diquat and other agricultural chemicals used in the control of aquatic weeds on the decline in apple snail populations of the Loxahatchee National Wildlife Refuge, Florida.

79. Occurrence of hepatomas in natural populations of fish and their relation to contaminant levels.

80. Discovery of exotic and other unknown contaminants in biota from critical habitats and from NPMP investigations: detection and identification of phenolic polynuclear aromatic and heterocyclic compounds by mass spectrometry.

81. Discovery of exotic and other unknown contaminants in biota from critical habitats and from NPMP investigations: expand methods for separation and analysis of unknown compounds by adaptation of highly efficient capillary chromatographic instruments.

82. Discovery of exotic and other unknown contaminants in biota from critical habitats and from NPMP Investigations: development of liquid chromatographic separation and detection methods for more water-soluble, relatively persistent pollutants not readily detected by gas chromatography.

83. Discovery of exotic and other unknown contaminants in biota from critical habitats and from NPMP investigations: uses of laboratory minicomputers for reduction of data from complex chromatographic separations and documentation of the occurrence of unknown and known contaminants.

84. Discovery of exotic and other unknown contaminants in biota from critical habitats and from NPMP investigations: develop analytical survey methods to measure (in fish, sediment, and water) inorganic pollutants from mine and particulate byproduct leachates derived from energy production.

85. Discovery of exotic and other unknown contaminants in biota from critical habitats and from NPMP investigations: development and implementation of sophisticated new mass spectrometry detection systems specific for phenols, and nitrogenous and other heterocyclic pollutants.

86. Biochemical analysis of intralaboratory samples from hazard assessment studies of toxic materials.

87. Biochemical analysis of samples from field monitoring studies for assessing contaminant-induced stresses on aquatic popultions.

APPENDIX C. ENVIRONMENTAL CONTAMINANTS EVALUATION - CURRENT WORK UNITS, AT SERVICE FACILITIES OTHER THAN THOSE AT ANN ARBOR, MI, AND COLUMBIA, MD

Seattle National Fisheries Research Center, WA.

1. Impact of contaminants from mineral and energy development on critical habitat for Northwestern and Alaskan salmonid and anadromous fish populations.
2. Determination of critical habitat requirements of Alaskan and Arctic fishes under overwintering conditions and their tolerance to environmental alterations.
3. Preparation of a manual of physiological methods for developing improved HSI equations for the FWS aquatic Habitat Evaluation Procedure for project impact evaluation.
4. Ecological impact of forestry practices on anadromous fish populations in southeastern Alaska.
5. Environmental tolerance levels of gas supersaturation in marine and alpine ecosystems.

Southeastern Fish Cultural Laboratory, Marion, AL.

1. Acute, chronic, and subchronic toxicity of organic contaminants to striped bass and other priority warmwater fishes and forage.

Cooperative Fishery Research Units

1. Georgia - Scanning electron microscope observations of gills of weakfish (*Cynoscion regalis*) and spot.
2. Georgia - Field acclimatization and laboratory acclimation of bluegills (*Lepomis machrochirus*) to pH.
3. Georgia - Adenylate energy charge as an indicator of stress in fishes.
4. Georgia - Changes in gill-tissue morphology induced by exposure to an anionic detergent.
5. Maine - Effects of Sevin applied to forests on food niche and growth of brook trout fry.
6. Maine -- RNA-DNA ratios as indicators of growth effects from insecticide exposure.

7. Maine - The ecological effects of log salvaging from reservoirs.

8. Maine - The ecological effects of log driving in relation to navigable rivers.

9. Maine - Population studies of intertidal fishes of the coast of Maine.

10. Ohio - Organochlorine contaminant levels in edible and inedible portions of selected Lake Erie fishes.

11. Ohio - Bilateral asymmetry in freshwater fish as an index of environmental contamination.

12. Oregon - Imprinting in salmon: Odorant recognition, effects of pollutants and artificial cues in saltwater.

13. Oregon - Chronic turbidity stress in juvenile coho salmon and steelhead trout.

Table 1.--Allantoinase activity of individual samples of frozen livers from wild lake trout collected in Lake Michigan off Saugatuck and Lake Superior off Isle Royale in 1974, and from Laboratory stocks of lake trout (± SE in parentheses). (From Passino and Cotant, 1979.)

Source, and number of fish	Total length (mm) Group	Total length (mm) Mean	Allantoinase specific activity (unit/mg protein)
Lake Michigan			
19	>500	707 (14)	0.0701[a] (0.00755)
18	<500	310 (26)	0.140[a] (0.0109)
Lake Superior			
18	>500	592 (10)	0.0712 (0.0103)
32	<500	350 (11)	0.0749 (0.00869)
Laboratory[b]			
3	>500	564 (6)	0.109 (0.0127)
30	<500	290 (14)	0.197 (0.0125)

[a] Difference between means of allantoinase activity within fish length group is highly significant ($P<0.01$) by analysis of variance.

[b] Fish maintained at Great Lakes Fishery Laboratory

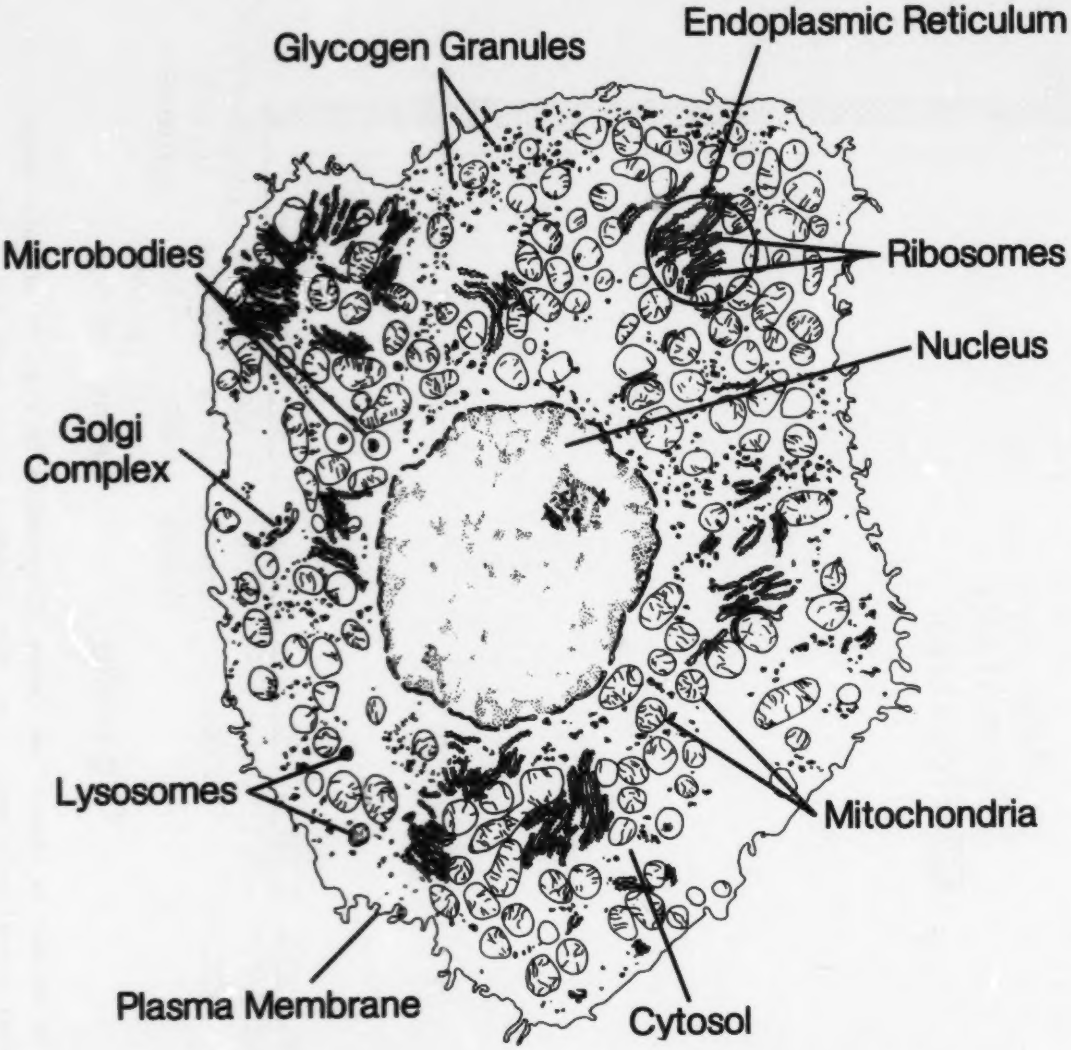
Table 2.--Contaminant analysis of composite samples of livers and individual samples of fillets of lake trout collected off Saugatuck in Lake Michigan and off Isle Royale in Lake Superior in 1974.

Source, and number of fish	Total length (mm)		Livers			Fillets
			PCB's	DDT+DDE	Total Hg	Total Hg
	Group	Mean ± SE	(μg/g)[a]	(μg/g)[a]	(μg/g)[ab]	(μg/g)[ac]
Lake Michigan						
10	>500	717 ± 23	21.4	4.68	0.939	0.594
10	<500	210 ± 14	<0.6	0.141	0.479	0.0552
Lake Superior						
10	>500	586 ± 14	4.55	1.86	0.690	0.581
8	<500	284 ± 4	<0.6	0.206	0.105	0.152

[a] μg/g wet weight.

[b] Average value of three (fish <500 mm long) or four (>500 mm) analyses per composite sample.

[c] Average value of two analyses per fillet for specified number of fish.


Glycogen Granules
Endoplasmic Reticulum
Microbodies
Ribosomes
Nucleus
Golgi Complex
Lysosomes
Mitochondria
Plasma Membrane
Cytosol

Figurel 1. Liver cell of the rat

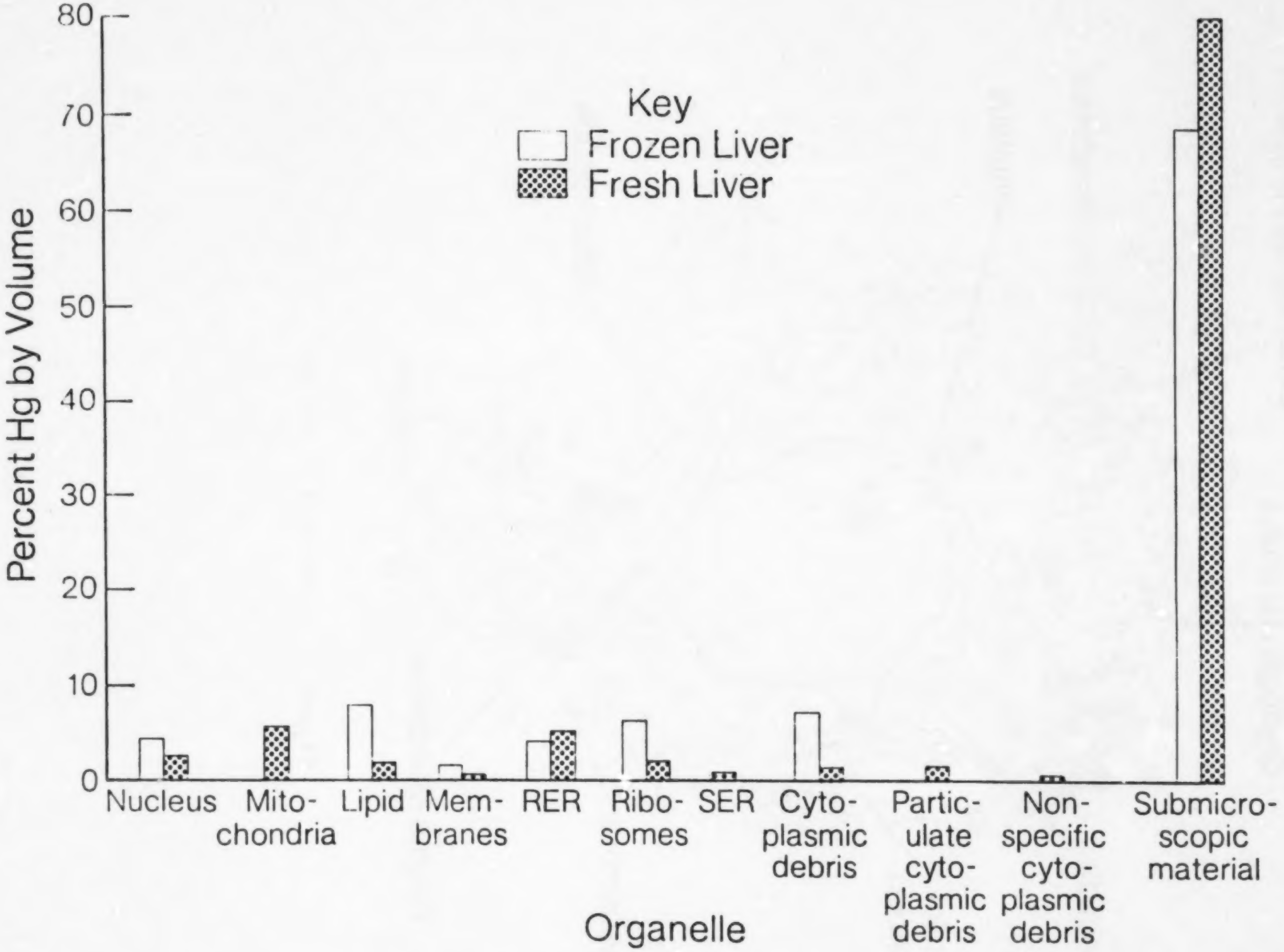


Figure 2. Percent mercury by volume in organelles (some illustrated in Fig. 1) of lake trout liver. RER = rough endoplasmic reticulum; SER = smooth endoplasmic reticulum. (D. R. M. Passino and J. M. Kramer, unpublished data.)

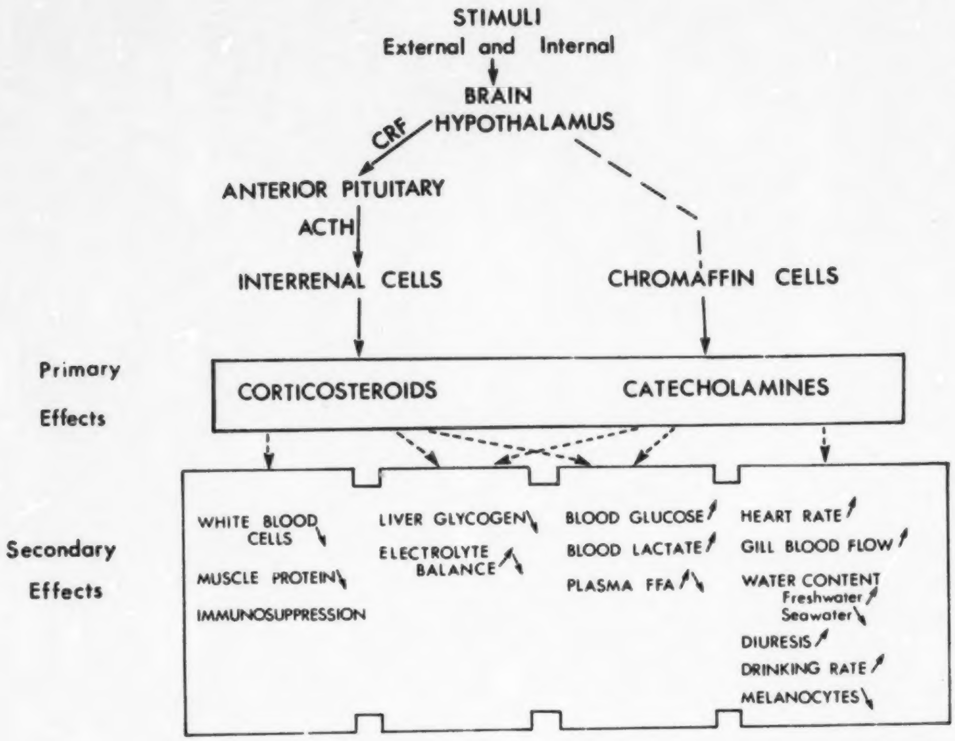

STIMULI
External and Internal
BRAIN
HYPOTHALAMUS
CRF
ANTERIOR PITUITARY
ACTH
INTERRENAL CELLS
CHROMAFFIN CELLS
Primary Effects
CORTICOSTEROIDS
CATECHOLAMINES
Secondary Effects
WHITE BLOOD CELLS ↘
MUSCLE PROTEIN ↘
IMMUNOSUPPRESSION
LIVER GLYCOGEN ↘
ELECTROLYTE BALANCE ↗↘
BLOOD GLUCOSE ↗
BLOOD LACTATE ↗
PLASMA FFA ↗↘
HEART RATE ↗
GILL BLOOD FLOW ↗
WATER CONTENT
Freshwater ↗
Seawater ↘
DIURESIS ↗
DRINKING RATE ↗
MELANOCYTES ↘

Figure 3. Scheme incorporating current information on the interrelationship between primary and secondary effects of stress in fish. (From Mazeud et al. 1977.)

OVERVIEW AND ASSESSMENT OF BIOLOGICAL SENSING

by

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INTRODUCTION

Biological in-situ sensors used to monitor indigenous terrestrial and aquatic biota could replace some of the more time-consuming and costly sampling and laboratory analytical techniques. Selective bioindicators and bioaccumulators are being utilized to identify potential exposure hazards before significant damage to the environment or human health occurs. Biological monitors can be used to identify toxic substances and to assess total environmental exposure to hazardous materials which have been originated for various sites. Terrestrial and aquatic organisms can function as in-situ monitors and remove much of the requirement for time-consuming and costly conventional monitoring techniques. Essentially, six approaches to biomonitoring are being evaluated at EPA.

1. In-situ bioassys

   Water, land, air

2. Bioaccumulation

   Aquatic organisms, terrestrial organisms

3. Indicator organisms

   Aquatic organisms, terrestrial organisms

4. Community and population changes (Species composition and abundance, biomass, diversity, productivity, mortality rates, etc.)

   Aquatic organisms, terrestrial organisms

5. Species health

   Tumor development, phenotypes, growth, reproduction, longevity, and stress measurements

6. Integration of biological methods to determine release of toxic substances from hazardous waste operations for

   a. Air,
   b. Water, and
   c. Land.

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Biological monitoring is neither a new concept nor a new activity. In fact, it was associated with the origins of current environmental concerns, that is, disappearance of desirable game fish from polluted rivers, accumulation of nuisance algae in eutrophied lakes, fish kills from pesticide runoff, and plant kills near smelters.

Recently, however, biological monitoring has received relatively less attention. This is for several reasons. One is the striking technical advances in our capability to detect and measure, by physical and chemical means, small amounts of contaminants in the environment. Another is the emphasis of the environmental protection laws enacted in the early 1970s on preventing the entry of pollutants into the environment without a need for direct proof of environmental damage.

Today we see a renewed interest in biological monitoring, which is the only means by which we can directly measure the degree of harm to man or an ecosystem resulting from environmental contamination or, conversely, the benefits from environmental protection.

The purpose of this paper is to examine biological monitoring in the context of current Agency needs - assessing the strengths and weaknesses of available biological monitoring techniques and attempting to define where opportunities do and do not lie for more effective and fruitful use of biological monitoring by the Agency.

WHAT IS BIOLOGICAL MONITORING?

The term "biological monitoring" can be applied to a rather large and diverse set of activities. It does not have a precise, generally accepted meaning; it means many things to many people. For this paper, it will be defined in terms of biological monitoring. This may be defined as the use of a living organism to measure, for a specific time and location, the presence or amount of a substance in the environment or in man, or the effect of a substance on the environment or man.

The keys to this definition are fourfold.

1. Use of a living organism

2. Specificity of time and location (to distinguish "monitoring" from "bioassay," such as the laboratory use of a fish or rat to determine an exposure/effect relationship)

3. Presence, amount, or effect in or on the environment

4. Relationship to a substance (Biological monitoring could be used to measure the environmental effect of a perturbation other than one resulting from entry of substance into the environment, e.g., destruction of fish habitat by stream channel modification. However, for this paper, the term is limited to effects resulting from a substance.)

There are three general approaches that can be taken to biological monitoring.

1. Use of an organism in the environment

This involves measurement with an organism in the environment to determine a presence, amount, or effect. The organism can be either naturally present or introduced.

a. Naturally present or indigenous organism (so-called "passive" monitoring)

Examples of this type of monitoring include human epidemiological studies, surveys of stream biota, and pesticides residue monitoring. Two general approaches exist for this kind of monitoring.

(1) Limited-species monitoring, where data are collected only from a single or several species. In this case, the organism selected is 1) the species to be protected, e.g. salmon, or is assumed either to be 2) particularly sensitive to the effect being monitored (e.g. eggshell breakage in ospreys due to DDT) or 3) to accumulate the contaminant of concern (e.g. radioactive iodine in animal thyroid tissue). In the latter two instances, the organism would be called and "indicator" or "sentinel" species.

(2) Community monitoring, wherein some feature of a biological community is measured such as its structure (e.g. which species are present in what numbers, species diversity, etc.) or its function (e.g. rates of energy flow, organic decomposition, biomass production, etc.).

b. Introduced species (so-called "active" monitoring)

In this case, the organism is intentionally introduced into the environment to take advantage of its sensitivity to an effect of concern or its ability to accumulate a contaminant. The classic example is the miner's "canary in a cage." More appropriate, as an example, is the use by the Germans of plants to detect air-pollution effects or accumulate heavy metals from air.

2. Use of an organism in the laboratory to measure a sample from the environment

Here the organism is kept in the laboratory, but the sample is collected from the environment. Examples include the use of fish bioassays to determine the toxicity of effluent samples and the recent attempts to use the Ames test to trace presumed carcinogens from industrial sources through the environment. It must be carefully noted here that "bioassays" are a biological monitoring technique only when they are applied to an environmental sample, and not when used to determine an exposure/effect relationship (e.g., LD50) for a specific chemical.

3. Use of a simulated environment ("microcosm") to measure either an effect or the movement and/or transformation of a contaminant

"Microcosms" are intentionally created, controlled assemblages of species of varying complexity and size (currently ranging from a jar that could be held in one's hand to very large "bags" suspended in the ocean) that are assumed to behave like natural ecosystems - at least for the functions being measured. Their unique advantage is their presumed capability to measure community (i.e., multispecies) phenomena under controlled, replicable conditions.

ASSESSMENT OF CURRENT BIOLOGICAL MONITORING TECHNIQUES

As mentioned above, there are many purposes for biological monitoring and many methods which have been used or are under development. The following is an attempt to describe the major types of biological monitoring and to assess their existing utility and scientific credibility and to identify what changes in biological monitoring capabilities are likely to occur in the near future.

1. Measurement of an effect using an organism in the environment, either indigenous or introduced

   a. Examples

      - Stream surveys which measure population size, species diversity, etc.
      - Use of plant species, either indigenous or introduced, to measure effects of gaseous air pollutants
      - Human epidemiological studies

   b. Types

      - Single or limited species that use indicator, sentinel, or sensitive species, either indigenous or introduced
      - Community or ecosystem structure or function

   c. Advantages

      The only way to measure directly a biological effect in the real environment. Such methods are most closely related to the real world, thus providing the most meaningful of all biological monitoring data.

   d. Disadvantages

      - Biological populations normally fluctuate widely and are affected by a large number of both natural and anthropogenic environmental factors. Thus, they are a rather insensitive measure of environmental contamination, reflecting only rather gross and fairly obvious changes. To detect changes, vast amounts of data may be required. Moreover, measurable populations are usually slow to respond to perturbation, often revealing change only when the damage is already extensive.
      - It can be very hard to define the cause of a biological change without considerable supporting data on exposures of environmental toxicants and other biological parameters. For example, it may be hard to

relate a decline in the size of a population to a pollutant, rather than to a change in another environmental factor such as a change in climate. Changes in community or ecosystem function can be measured, but the ecological significance of relatively small changes are not well understood. Certainly, the public and government officials have not been presented a persuasive case for the significance to human welfare of small changes in ecosystem functions, such as energy cycling.

- Indicator or sentinel species present special problems. Their response can vary considerably with the age, sex, and genetic background of the organism. Therefore, the advantage of an indicator organism, namely collection of data on a single species rather than the whole system, can be illusory; considerable data may be needed from the system to interpret the data from the indicator species. In order to choose an effective indicator species, the chemical of concern must be known and, often, its behavior and the nature of its effect on the environment as well.

e. Assessment

General biological surveys which measure population sizes or other aspects of community structure or functions are usually too data-intensive to be useful for purposes other than determining the gross state of health of an ecosystem. Cost-effective examples of the type of survey are hard to find. For most cases which have been examined, the major deficiency has been the lack of a clearly defined objective - either a testable hypothesis or a specific range-finding goal to guide data collection. Even when the survey has a clear objective, the amount of data required to reveal a measurable change of significance may be impracticably large.

Use of biological effects data for general trend monitoring seems to be even more impractical at this time. Without enormous amounts of data most trend monitoring nets are too coarse to detect effects at a useful level or in a useful time frame. If biological effects are to be used for trend monitoring, this should be done only for very specific and carefully targeted purposes. The system must be very carefully designed and evaluated for cost-effectiveness ahead of time.

The most promising and underutilized approach to effects-monitoring appears to be active monitoring, using introduced species. This approach offers a high degree of sensitivity, reliability, and precision of data. Since many of the variables associated with the organism itself are controlled, the effects measured can be more easily attributed to environmental factors. A certain amount of accompanying data on the ecosystem are necessary to interpret changes occurring in introduced species, but the amount of data may be considerably less than necessary for indigenous species. Use of an appropriately sensitive introduced organism could provide a real time detection or alarm system for environmental chemicals, as well as a relatively reliable means for measuring change in environmental stress.

Active monitoring or use of introduced species has not been widely used in this country. The Germans and Japanese appear to have developed a number of such systems. The basic scientific capability exists for this approach to monitoring and, with an intensive effort, such systems could be developed and validated.

Most effects-monitoring has involved acute toxicological effects on a sentinel organism or actual changes in numbers within a population. However, several recent approaches offer promise of measuring effects prior to actual death of the organism. These are (1) measurement in the laboratory of sublethal changes, such as physiological responses or states, or the behavior of organisms taken from the environment and (2) use of plant mutation rates to detect the pressure of genetically active chemicals in the environment.

2. Measurement of the presence or amount of a contaminant in the environment or in an organism

a. Examples

- Measurement of pesticide residues in fish, shellfish, birds, and human tissue
- Monitoring of radionuclides in the human food chain (e.g., cow's milk) or in accumulator organisms or tissues (e.g., ^{131}I in animal thyroid tissue)
- Use of plants in Germany to indicate the presence of heavy metals in air
- The U.S. Mussel Watch program
- Measurement of metals (e.g., lead) in human hair as an indication of exposure (e.g., use of Isotope Zeeman Atomic Absorption Spectrometer)

b. Types

- Measurement in a human food chain organism to indicate the existence and potential magnitude of human exposure to a chemical
- Measurement in an accumulator organism (which need not be in the food chain) to detect the presence of a chemical in the environment, and possibly, to measure its environmental level
- Measurement in a sampler or integrator organism (i.e., one which does not bioaccumulate the chemical of concern, to detect the presence of a chemical in the environment, and possibly, to measure its environmental level)
- Measurement of the chemical within the affected organism as an indication of actual exposure

- Specimen banking, which could involve any of the above, but with storage of the sample for deferred analysis (This allows retrospective analysis for chemicals which were not of concern at the time the sample was collected, but have become of concern subsequently.)

c. Advantages

- When bioaccumualtion occurs, this approach may be more sensitive than physical/chemical methods. Essentially, the organism is used as an efficient "trappng" system.

- Since the actual presence of chemical in an organism is measured, these data provide greater significance as to biological relevance. In some cases, such data can provide a reasonable measure of either the potential exposure in the food chain or the actual amount of chemical reaching and being taken up by the organism.

d. Disadvantages

- One usually must know ahead of time which chemical to look for (except for specimen banking) in order to select the appropriate organism for sampling. This is because the uptake or accumulation may vary considerably according to the chemical or the organism involved. We are not able at this time to define sentinels for a broad spectrum of chemicals.

- Data on indigenous species can be highly variable, due to different responses with age, sex, and genetic differences.

- Trend monitoring nets are usually either too coarse or too costly. For example, market-basket surveys have not been especially effective in detecting agricultural chemicals in food, since most exposures of concern are due to spills, inadvertant misuse, or other intermittent or local incidents. However, trend monitoring can be useful for measuring gross environmental changes, such as the widespread decline in amounts of DDT present in fish or shellfish, or for highly focused, local surveys.

- Methods for reliability sampling and analyzing chemicals in living tissue are not generally available.

e. Assessment

Use of living organisms to measure chemicals in the environment or actually within an organism of concern (e.g., human tissue) is currently feasible. The keys to success are (1) knowing the chemical of concern and enough about its behavior and effects to be able to select an appropriate organism for sampling, (2) controllng the variability within the organism itself (using a standard, introduced organism is a very promising way to achieve this), (3) allowing for the necessary, and sometimes tedious refinement and validation of chemical analytic methods, and (4) clearly defining a realistic objective, i.e., selecting changes of concern that can be detected within the time and sensitivity required by a system of reasonable cost. If designed properly, such systems can be very effective, for specific chemicals, as chemical detectors or measures of potential or actual exposure.

Use of such systems for measuring trends over a broad area or for many substances is most likely to be impractical except for rather limited purposes such as the measuring changes in general environmental levels of pervasive chemicals such as DDT or PCBs.

The two most promising developments with this approach to biological monitoring appear to be (1) use of introduced species and (2) measurement of actual amounts of chemicals in human tissue as an indication or even quantitative measure of actual exposure.

3. Laboratory analysis of environmental samples

a. Types

- Use of toxicity or another general biological parameter as a performance requirement for a pollution control system, e.g., inclusion of toxicity as measured by fish bioassays as a performance requirement for wastewater treatment systems.

- Use of a biological-effects parameter, such as a genetic effect, as an indication of contamination of the ambient environment or a means for tracing contributing sources.

b. Advantages

- Use of a single, general parameter can be considerably cheaper than chemical analysis since it does not require measurement of a possibly large number of chemicals.

- This approach can detect effects of unknown chemicals as well as synergism or antagonism among several chemicals.

- The data are highly precise, reliable, and sensitive. In addition, bioassay data have historical credibility for regulatory activities.

c. Disadvantages

- It can be very hard to relate cause and effect. If the toxicity performance limit is exceeded, further data are needed to define the causal agent.

- Each technique measures only one stress, for instance acute toxicity. Several assays may be needed to cover the major stresses of concern.

- Such an approach does not normally account for bioaccumulation.

d. Assessment

General toxicity, such as measured by a fish bioassay, has considerable promise for use now as a regulatory tool. It can be reliably and conveniently done, and can be considerably cheaper than chemical analysis. It has certain limitations: the causal factor is not defined, bioaccumulation is not considered, and reflects only the single stress of toxicity. However, it may provide a reasonable and practical surrogate for a broader spectrum of effects.

4. Measurement of fate and/or effect in laboratory ecosystems - microcosms

Microcosms, or controlled ecosystems, is a term which applies a rather wide range of experimental approaches to reducing the enormous number of variables in the real environment while preserving essential interactions among components of a real environmental system. The microcosm need not simulate the real environment in all aspects, but only those of concern for the experiment. Its advantage is the ability to simulate systems effects or processes and not just those of a single species. Microcosms vary from rather small, multispecies assemblages in a jar that could be held in one's hand to rather large attempts to capture and control segments of the environment, such as an artificial stream channel or the suspension of a massive, semienclosed bag in the ocean.

Because microcosms vary so widely in nature, purpose, size, and cost, it is hard to speak generally of their current or future possibilities. None appear to have reached an operational stage of utility; thus, for now, they remain research techniques. However, they offer considerable promise as a means for measuring both environmental effects and environmental processes, such as transport and fate. Their ability to simulate, usefully, the real environment needs to be determined as soon as possible.

CONCLUSIONS

As pointed out earlier, biological monitoring provides a nearly-unique advantage in the measurement of real effects on the living environment, which has led recently to a renewed interest in this technique. The search for real or meaningful data has led most past efforts at biological monitoring in this country to concentrate on measurements using indigenous organisms. These efforts have not been notably cost-effective. Because of the large natural fluctuations in environmental systems and the large number of variables affecting these fluctuations, such approaches to biological monitoring, although the most meaningful, are imprecise, insensitive and very hard to interpret.

One way to get around the complexity and variability of natural systems is to monitor so-called indicator species. However, the advantage of simplicity in using indicators can be illusory. Often the overall environmental system has to be understood in order to select an appropriate indicator organism or to interpret the data resulting from its use.

Thus the direct monitoring of effects using indigenous organisms is severely limited in practicability and utility. This is not to say that such activities are totally impractical or useful. They have a definite place for measuring gross environmental conditions or changes or for intensive, targeted (and usually costly) local surveys. They appear not to be promising for widespread, routine use at this time.

This is especially true for broad environmental-effects screening to identify problems otherwise missed or trends that allow us to anticipate problems. Because of the variability of natural systems, the data needed to detect effects of changes in environmental systems with a useful degree of resolution would be enormous, and probably not worth the cost of collecting.

Are there any opportunities for more effective use of biological monitoring for effects? The major promise appears to be with the use of introduced organisms - so-callled active monitoring. This approach appears to be used effectively by the Japanese and Germans.

Use of introduced organisms can be less appealing scientifically, since it is a way of getting around having to understand natural systems, and it has some disadvantages in interpreting the meaning of the data. Nonetheless, it offers real promise for detecting and measuring environmental effects of chemicals and should be aggressively analyzed and, if need be, developed for use in this country.

Biological monitoring for the presence or level of a chemical in the environment offers great promise for specific chemicals. Its successful use in the past has always, apparently, been associated with a specific chemical of concern: a radionuclide, a pesticide, or a heavy metal transported by air. As described above, its advantages are (1) greater biological relevance, (2) greater sensitivity, and (3) ability to indicate actual or potential exposures. Techniques either are available, under development, or can be relatively easily developed. As the Agency moves towards regulation of specific chemicals, this biological monitoring technique should prove very useful and cost effective. Again, the key is to monitor for a specific, known chemical. The possibilties are numerous: a detector, an alarm, a measure of food-chain levels, a measure of actual exposure.

The use of a general toxicity measure as a performance requirement for environmental control systems, such as industrial wastewater treatment, seems to offer sufficient promise that it is puzzling why it has not found broad favor. Reluctance appears to result from concern that a general toxicity measure, such as a fish bioassay, does not identify the causal agent and does not detect certain effects, such as carcinogenicity. Nonetheless, this approach seems to warrant more serious consideration, expecially if linked in the future with other bioassay techniques, such as those for genetic effects.

One anomaly became apparent during this analysis. So far, most environmental attention appears to have been given to monitoring of aquatic systems, except for some work on pesticides and on terrestrial effects of air pollution. It seems as we move more to a concern over human health effects from environmental contaminants and protection of our environmental life support system that relatively greater attention is warranted for terrestrial systems, including soils. Our efforts on biological monitoring should reflect this shift in emphasis, particularly for future monitoring of hazardous waste sites.

NEW IN-SITU TECHNIQUES IN BIOMONITORING

by

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INTRODUCTION

Continuous in-situ biological monitoring is essential to the understanding and improvement of water quality because these systems continually assess the responses of selected indicator organisms to the changes occurring in the water chemistry and physical parameters and are much less expensive than ecological surveys.

DETERMINING WATER QUALITY

Successful predictions of water quality changes can be determined with biomonitors in-situ depending upon the selection of indicator organisms, membrane filter selectivity, and the measurement of pertinent physical and chemical data at the test sites. Biomass increase and the health of the organisms within the biomonitors in-situ over 3- to 30-day periods reflect the degree of eutrophication and the presence of toxic compounds such as pesticides, herbicides, heavy metals, and radionuclides. Bioconcentration of these substances within test organisms such as Hydrodictyon, Pithophora, Rhizoclonium and cultures from the Unialgal Pollution Indicator Set (Carolina Biological Supply Company 15-1550) indicates the toxicity of these substances to the test organisms in-situ and their relative abundance in the water.

Species diversity studies using mixed populations can be conducted with biomonitors in-situ. Biomonitors are also ideally suited for studying possible morphological and physiological changes in species under different environmental conditions in nature and in the laboratory. Equilibration time, i.e., when the water chemistry within the biomonitor approximates that of the surrounding water, requires 24 to 48 hours, varying with the water quality. Procedures for using biomonitors (sterilization, types of media, test orgnisms, equilibration time, and bioassay techniques have been previously described (Schlichting, 1976).

DILUTE MEDIA DUPLICATES NATURE

Trainor and Shubert (1973) suggested that algae to be used in bioassay procedures should be grown in a dilute medium which more closely duplicates conditions existing in nature. Daily transfers of algal cells to fresh dilute media supported good growth in their laboratory studies. Growing algae in dilute media within a biomonitor situated in the precise water area under investigation for the time period desired eliminates the need for daily transfers. The nutrients and metabolic products are recycled by diffusion processes and mixing occurs from chamber agitation by the currents as well as biological pumping within the biomonitor.

The composition of most defined media generally does not resemble that of natural waters and can even bring about morphological changes in the organisms under study. It is recommended that test site water, sterile filtered with 0.2 um porosity filters, be used to prepare dilute media for culturing test organisms and filling the biomonitors. Dilute media, more closely approximating the mean chemical composition of river water in parts per million, would be: carbonate 58.4, calcium 15, silicon 13.1, sulfate 11.2, chloride 7.8, sodium 6.3, magnesium 4.1, potassium 2.3, nitrate 1, and iron 0.67 for a total of 120 ppm (Livingston 1963).

PREDICTIVE VALUES USING ALGAE AS INDICATORS

Algal growth potential (AGP) is defined as the organic mass resulting from a 7-day incubation of a culture grown in a natural water substrate under standardized laboratory conditions and expressed as mg of dry organic mass per liter of sample (Wang et al. 1973). A similar definition could apply to the growth of microalgae in biominitors in-situ for the same unit of time under various diurnal and seasonal environmental conditions. Thus biomonitor studies of a body of water over one year's time would give a good indication of the AGP for that given body of water and should have high predictive value for discerning future water quality changes.

By growing algae in-situ within a biomonitor, grazing is eliminated and the organisms can respond to all the physical and chemical factors at the test site. The degree of growth and health of the algae per unit time will indicate water qualtiy (Mason 1978). The comparison of the growth of Anabaena within biomonitors placed one foot below the surface in Lake Itasca and in a sewage lagoon showed that growth was nearly twice as great in the lake as in the lagoon over a two-week period. (See table 1.) With higher nutrient content, alkalinity and total hardness in the lagoon, a higher growth rate would be anticipated. However, a greater turbidity and a mat covering the lagoon surface reduced the available light actually retarding growth. The results of the study demonstrated the importance of recording both chemical and physical parameters to better understand the experimental results.

On the basis of this study, it is recommended that the following additional data be recorded in Biomonitor studies: ammonia nitrogen, specific conductivity, total dissolved solids, total suspended solids, nitrite nitrogen, light penetration, and meteorological conditions (Mason, 1978).

STUDIES USING THE BIOMONITOR

Biomonitors are suitable for many other types of studies. Radionuclide bioconcentration in the aquatic environment as well as the degree of toxicity of sewage effluent, heavy metals, pesticides, herbicides, growth regulators, and

Table 1.--Growth of *Anabaena* in situ over a two-week period

Parameters	Lake Itasca	Sewage Lagoon
Increase in biomass (Inoculum 82 mg)	298 mg	121 mg
Temperature range	20.5-24.5°C	19.8-26.5°C
Nitrogen range	0.02-0.04 mg/l	0.06-0.16 mg/l
Orthophosphate	0.25-0.30 mg/l	5.50-9.60 mg/l
pH	9.2	9.1
Alkalainity range	165-168 mg/l $CaCO_3$	343-366 mg/l $CaCO_3$
Total hardness	141-143	282-285
Turbidity range	3-5	10-27

agricultural fertilizers to the indicator organisms in strip mine ponds, lakes, and rivers can readily be studied. These instruments are also suitable for environmental impact studies leading to the reclamation of waterways for public use. Species diversity studies of mixed plankton populations utilizing the Palmer Index (James 1975) and studies of possible morphological and physiological changes in species under different environmental conditions can also be determined by biomonitor in-situ studies. The incubation of phytoplankton in situ in the sea or lakes, although inconvenient, should be done more frequently (Fogg 1965).

The great variety of studies in which biomonitors may be utilized to biologically monitor "nature's variables" is limited only the ingenuity of the researcher.

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PROCESSING OF ZOOPLANKTON SAMPLES BY ELECTRONIC IMAGE ANALYSIS[1]

by

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INTRODUCTION

Methods of identifying and counting zooplankton in preserved samples have changed little since Johannes Muller first towed a fine-mesh net through the ocean more than 100 years ago. Instrumentation basically consists of a microscope and a ruled counting chamber. Because time of analysis for a single sample ranges from hours to days, backlogging is inevitable.

We are attempting to modernize the procedure, using improved image formation devices and computerized pattern recognition techniques. Thus far, total counts and size-frequency distributions can now be made in minutes with a simple, processor-controlled vidicon system, but calibration remains a problem awaiting sharpened contrast by optical edge enhancement and spatial filtering.

Major representatives of North Atlantic zooplankton can be accurately classified to group (e.g. copepods, fish eggs, fish larvae, cladocerans, chaetognaths, and euphausiids) by discriminate analysis of simple morphometric relations (length, width, perimeter, and area). Discriminate analysis results of 93 well defined hand-traced zooplankton silhouettes resulted in 100% successful identification. When the same analysis was performed on 240 images of real zooplankton, the results, although not perfect, were extremely encouraging.

1. A technological bottleneck--We are solely dependent on manual operations for sorting of preserved zooplankton samples, even though physical fractionation procedures exist. A range of electronic approaches has been considered (Sutro 1974, Almeida et al. 1977), and particle discriminators work in limited ways (Beers 1976, Fawell 1976, Uhlmann, et al. 1978). But the task is so arduous

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that few technicians can work continuously at counting, identifying, and measuring organisms under a microscope. Personnel turnover is understandably high, and training periods further reduce sample output. Consequently, effective average processing time (per technician and year) ranges up to four days per sample.

The resulting bottleneck means that sampling designs meeting modern-day statistical requirements are impossible. The MARMAP program alone has a capability for collecting 4,500 samples annually (Sherman 1978) which is probably near the world capacity for sample processing. Clearly then, a technological breakthrough is required to support basic research in plankton biology and to meet the requirements of improved fisheries forecasting. We are addressing the problem with tools that have been developed in widely disparate fields. The reuslts are encouraging, but they do show the need for engineering advances in selected areas.

Pattern recognition--Among the several approaches applicable to analysis of samples collected with zooplankton nets, we believe that pattern recognition by combined optical and electronic means is the most promising. In the sense used here, pattern recognition consists of counting particles, extracting visual features from individual organisms (either alive or preserved), measuring these features automatically, classifying the organisms into ecologically useful and taxonomically meaningful categories, and finally giving vital statistics of length, area, perimeter, etc., for each group. Classification to species is the ideal, and our work on morphometrics indicates that it may be easier than one would imagine, but, for present purposes, we have established the following groups as technologically amenable to feature extraction and classification:

- Copepods (copepodite stages I-VI)
- Nauplii (various)
- Zoeae (Brachyura)
- Zoeae (Macrura)
- Mysids, cumaceans, euphausids
- Amphipods
- Fish eggs
- Fish larvae and chaetognaths
- Lamellibranch larvae
- Polychaete larvae
- Medusae
- Particulate debris, organic aggregate

From total count and size-frequency distributions within these groups, useful descriptors of ecosystem state can be derived (Steele and Frost 1977).

APPROACHES TO PATTERN RECOGNITION

The components of a pattern recognition system suitable for preserved zooplankton samples consists of (1) a low power microscope or enlarging lens, with provisions for transmitted, incident and dark field illumination; (2) vidicon and input image monitor; (3) analog-to-digital converter; (4) minicomputer; and (5) output image monitor and line printer. (See fig. 1.) Figure 2 shows our image processing system in more detail. The minicomputer is programmed to extract and measure specific characteristics. These features best characterize a large, but dimensional and finite, pattern space that has been reduced by the system's transducer (optical-vidicon system). The next step is a classification function that makes decisions for assignment of the input object to one of the predetermined classifications. (See fig. 1, Fu 1970.)

1. Mathematics of pattern recognition--The mathematics of pattern recognition vary from comparatively simple "template-matches" stored in the computer's memory to complex scene analyses consisting of so many features and patterns that the analysis must be described in hierarchical order from simpler subpatterns. The decision-theoretic approach, which discriminates objects according to statistical models, has been most commonly used. In a statistical sense, " . . . each pattern is considered as a vector in n-dimensional space. The goal of the recognition system is to define partitions in this space such that each region can be identified with a class of pattern." (Viglione 1970). In the future, capabilties of the syntactic approach to description of structural pattern should become popular, especially for identifying biological objects (Fu 1978). Chromosome analysis, for example, has been automated by way of syntactic grammars. Our work to date has been limited to the discriminate-function approach, and although the classification ability for planktonic animals is surprisingly good, the two approaches will undoubtedly be combined in a semiautomatic instrument for classification, enumeration, and sizing of planktonic organisms.

2. Image formation by electronic means--In figure 3, the computer-generated outline of an adult female *Centropages typicus* viewed directly by vidicon is compared with a manmade drawing traced from the input monitor and then resubmitted for computerized imaging. Note that edge definition of what man sees and interprets is far sharper than the computer generated pattern of an actual specimen. Herein lies the problem, much of it arising from the simple biological fact that low contrast makes planktonic animals hard to see. This is certainly adaptive for organisms swimming in nature, but the problem it causes for us may be solved by such refinements as a laser beam directed at individual specimens and spatial filtering of the coherent image at the frequency plane.

A second problem shown in fig. 3 (lower) is the orientation of dead specimens. Because their density is only slightly greater than neutral, they fall in unstable positions. The left specimen appeared to have an asymmetric urosome, but this was not so; rather it was a visual aberration due to the copepod lying at about 45° on its major (length) axis, balanced tenuously on its plepods, which in this case happened to be extended.

As shown in fig. 4, computer-generated images from silhouettes can be manipulated in various ways. Length, perimeter, width area, etc., of up to 5 organisms per field to view have been obtained. Figure 5 (top) shows the computer-generated image of a preserved crab larva and its smoothed verion superimposed on it. The lower part of the same figure shows the same zooplankton with smoothed silhouette. A copepod with its smoothed version is also shown. The smoothed perimeters have considerably less number of points and thus the time it takes the computer to extract the necessary features is shortened considerably.

To complement the above electrooptical approach, we have also explored applications of silhouette photography (Ortner et al. 1979). Images shown in fig. 6 may be better for input to currently available vidicons than are the organisms themselves, assuming, that a well focused image can be achieved. An advantage here is that this lensless system of direct photography by electronic flash can be done at sea with live organisms. Alive, they orient in rather repeatable ways from one individual to the next, but in a preserved sample random position is a problem that must be solved, possibly with a two-camera system trained on organisms hydrodynamically oriented in a flowing, as opposed to static, observation medium.

RECOGNITION OF ZOOPLANKTON

1. Silhouette Identification of Zooplankton--To establish a reference data base free of electronic-imaging problems, we photocopied 93 published plates of different species, chiefly from Fishes Identification by Zooplankton of the Conseil International pour l'explortion de la mer. These species represented 5 of the 12 categories listed above. The groups which were represented included copepods, chaetognaths, euphausids, fish larvae, and cladocera.

The variables used in the classification were perimeter (S_1), square root of area (S_2), the maximum length (S_3), and the maximum width (S_4). Since one purpose of this work was to produce a classification process independent of the actual size of the images, 6 "normalized" variables were produced from the four originals. These were

$$X_1 = \frac{\text{perimeter}}{\text{square root of area}} \qquad X_2 = \frac{\text{perimeter}}{\text{maximum length}}$$

$$X_3 = \frac{\text{perimeter}}{\text{maximum width}} \qquad X_4 = \frac{\text{square root of an area}}{\text{maximum length}}$$

$$X_5 = \frac{\text{square root of an area}}{\text{maximum width}} \qquad X_6 = \frac{\text{maximum length}}{\text{maximum width}}$$

Figure 7 shows a typical clustering of the silhouettes on a two-dimensional feature space $X_2 - X_4$. The numbers in the field of this diagram refer to the previously defined taxonomic groups, e.g., 1 = copepods. Although the clusters appear distinct, the cluster algorithm did not give accurate results. Therefore, we ran a discriminate analysis test (SAS program) using 93 organisms as a training test with a test set of 15 observations. The results showed perfect score by identifying all 15 animals.

However, it must be pointed out that the perimeters of the animals were well and clearly defined with minimum additional electronic noise disturbing body outlines. In addition, the several silhouettes from the same species were similar in shape although they were different in size and orientation.

2. Identification of plankton--Two-hundred and forty animals of six groups (group: 1 - copepods, 2 - zoea, 4 - euphausid and mysid, 6 - fish eggs, 7 - fish

larvae, and 8 - pteropods-bivalve) were digitized and their edges were extracted. From the perimeters of these observations, additional features were extracted, which were (a) seven invariant moment functions, each one being a function of several moments; (b) the ratio of widths at one-third distance from the two edges of their longest dimension; and (c) circle similarity (a feature to differentiate circular animals, e.g., eggs, from any other group).

From the 15 features, we selected 11. These were the 6 features which they were used to discriminate the silhouettes, three invariant moment functions, the 1/3 distant ratio and the circle similarity feature. Based on these 11 features, we ran discriminant analysis using SAS programs. Figure 8 shows a two-dimensional characteristic distribution of zooplankton on the feature space $X_2 - X_4$.

Figure 9 shows the results of discriminant analysis. The training sample in this case was consisted of all 240 observations (animals). The large misclassification error for groups 6 and 8 is due to the extreme similarity of the observations. Otherwise, the classification success shown in fig. 9 is very encouraging.

Figure 10 shows results of discriminant analysis when the training sample was consisted of 200 observations (animals) and one test set of 40 observations.

Figure 11 shows results on electronically smoothed body outlines which indicate that the success of classification is sensitive to smoothing. This is understandable, since smoothing tends to round long shapes and tends to elongate the round shapes. Additional studies are needed to create a more efficient algorithm such that the smoothed animals retain their identifying detail characteristics.

Figure 12 shows mean misclassification error. These results are based on six different samples from the original and six from the smoothed animals. Each sample had one training set of 200 observations and one test set of 40 observations. The mean misclassification error of the smoothed animals was somewhat higher, as was anticipated from the results mentioned above.

In addition to the discrimination results, the automated analysis will provide far more needed information than human operators may provide within any reasonable time and effort. Vital statistics of area, perimeter, maximum lengths, and maximum widths for each group will be easily obtained. Figure 13 (upper part) shows the distribution of (square root of area)/(maximum width) and the lower part of the same figure shows the distribution of (maximum length)/(maximum width) of the group 6. Figure 14 shows the distribution of the first invariant moment of group 6. In this initial study, we have not kept the information about the microscope magnification used for each animal and thus any distribution of a single feature would have been meaningless. However, in our future studies this information will be included and distributions of the desired variables will be provided.

MEASUREMENTS WITH BAUSCH AND LOMB QMS SYSTEM

Lengths of 300 *Calanus finmarchicus*, copepodite stages IV-VI, were measured once manually and twice with the Bausch and Lomb QMS system (Maurer 1978). The machine consistently measured 0.1 mm lower than a technician through both runs, but upon adjustment for systematic error, the manual and electronically determined distributions, compared graphically in fig. 15, became statistically identical (Kolmogorov-Smirnov test). Furthermore, repeatability of machine measurements was extraordinarily high (P 0.01), which is much better than would be expected by any one or two technicians.

A comparison of the times required for counting and length measurement by manual and by electronic means was made "pitting" an experienced technician against the QMS system. Five samples were prepared, each consisting of 25-100 stage V-VI *Calanus finmarchicus* copepodites, selected to yield a unimodal size frequency that would readily reveal minor deviations. The technician used a stereo microscope at 12x magnification, calibrated in the usual way with a stage micrometer. Copepods for electronic analysis were stained for about 16 hours (overnight) in a dilute solution of methyl blue to enhance image contrast. A specially made template of elipses in the general range of copepod lengths was used for calibrating the QMS system; detrital particles were rejected by the operator dialing a minimum size criteria; samples with up to 500 individuals required counting 10 successive fields. Operator control was insured by an enhanced outline automatically drawn around each object detected for measurement.

Electronic counting was very fast, taking less than 0.1 second for a sample of 100 organisms. Manual counting required 0.7 min and measurement 40.2 min. Total time for all operations - from sample preparation through counting, data summation and plotting a size-frequency distribution - was 53.5 min manually but only 11.9 min by the image analysis system, for a saving of 78%.

Manual processing of a 500-organism sample required 264.2 min vs 72.1 min electronically for a 78% saving. Elimination of plotting the length distribution would reduce manual analysis by 36 min, but data summation is a necessary step and by hand it alone takes 21.0 min.

Even with a more elaborate image analysis than the QMS system, time savings would probably not exceed 85% over manual. Sample preparation still required up to 25 min for a 500-organism sample (examined as a series of subsamples in a multi-welled chamber).

With new devices for sample preconditioning and automatic scanning, we should be able to count, measure, and group 500 organisms in 5-6 min. With allowances made for downtime and systems upkeep, this translates to about 5,000 samples per year, which is near the requirements of large-scale projects. Perhaps more important is the simple fact that any ecological science draws its very substance from a continuing census of populations in nature. Although this seemed impossible for plankton biology a short while ago, the way to go is now clear.

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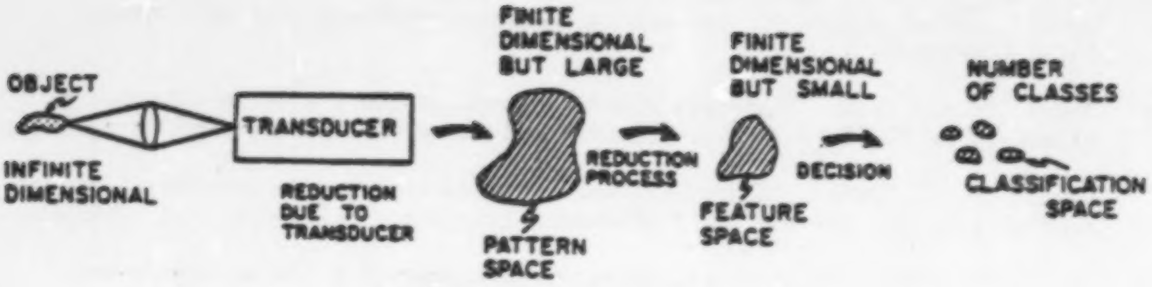
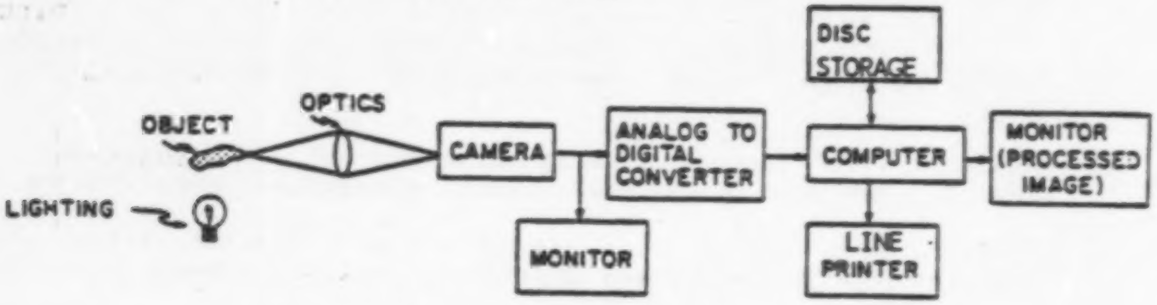

FINITE DIMENSIONAL BUT LARGE
FINITE DIMENSIONAL BUT SMALL
NUMBER OF CLASSES
OBJECT
TRANSDUCER
INFINITE DIMENSIONAL
REDUCTION DUE TO TRANSDUCER
REDUCTION PROCESS
DECISION
PATTERN SPACE
FEATURE SPACE
CLASSIFICATION SPACE

CONCEPTUALIZED


DISC STORAGE
OPTICS
OBJECT
CAMERA
ANALOG TO DIGITAL CONVERTER
COMPUTER
MONITOR (PROCESSED IMAGE)
LIGHTING
MONITOR
LINE PRINTER

TYPICAL COMPONENTS

Fig. 1. Typical electronic recognition system.

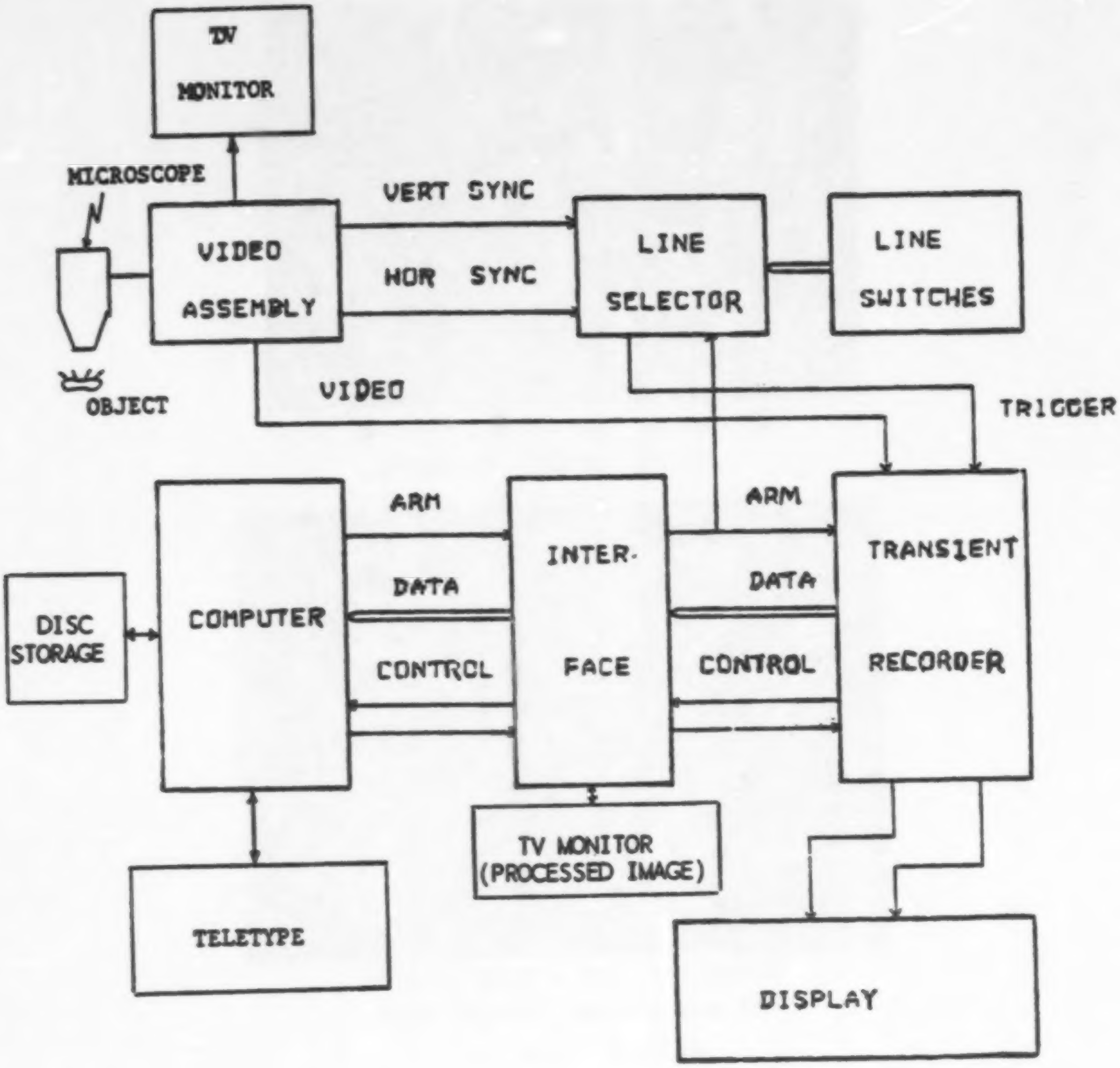
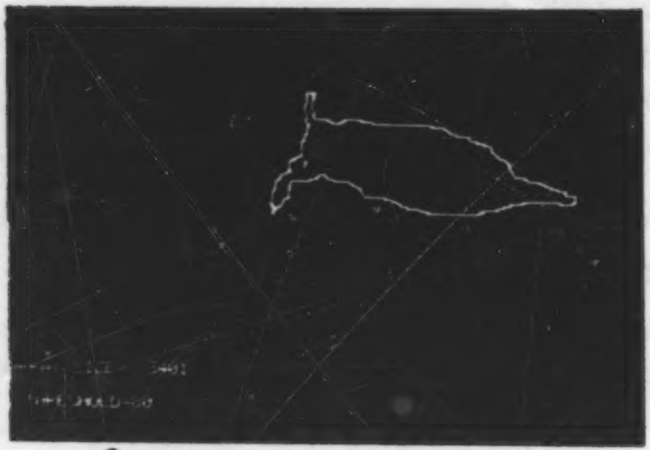
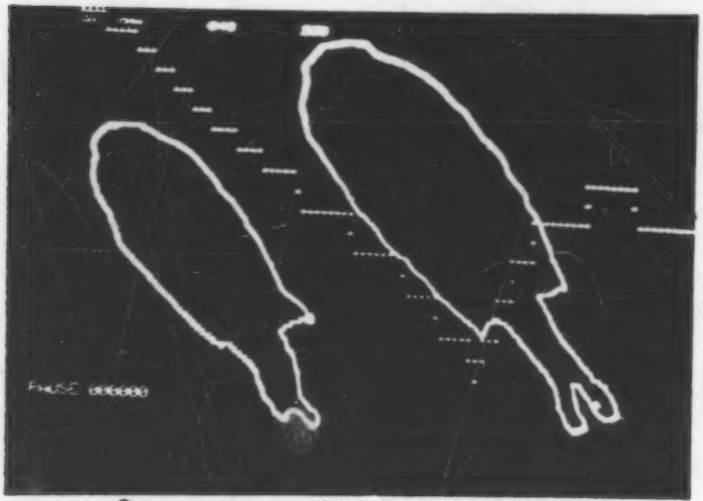

TV MONITOR
MICROSCOPE
OBJECT
VIDEO ASSEMBLY
VERT SYNC
HOR SYNC
LINE SELECTOR
LINE SWITCHES
VIDEO
TRIGGER
ARM
DATA
CONTROL
ARM
DATA
CONTROL
DISC STORAGE
COMPUTER
INTER-FACE
TRANSIENT RECORDER
TV MONITOR (PROCESSED IMAGE)
TELETYPE
DISPLAY

Fig. 2. Details of experimental setup.

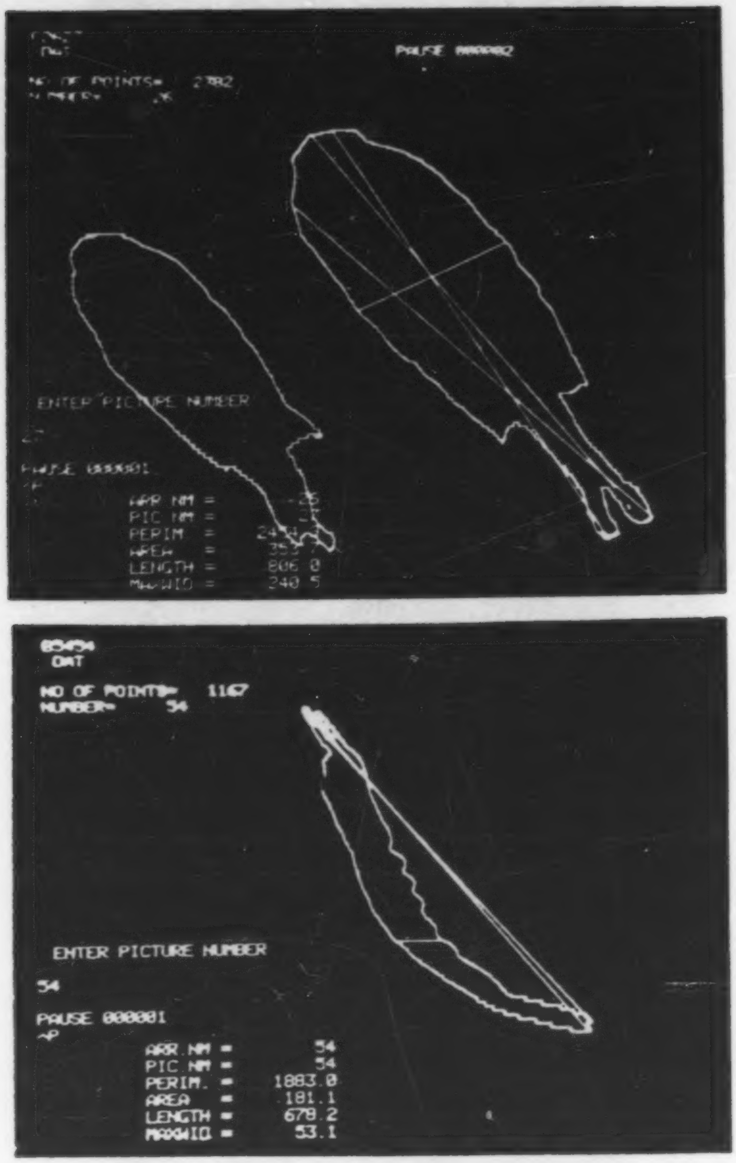


Computer generated image
of an actual copepod.



Computer generated images
of silhouettes traced from
vidicon monitor.

Figure 3. Computer-generated images of real and silhouette plankton images.



```
[illegible]
DAT

NO OF POINTS=   2782
NUMBER=      26
                                    PAUSE 000002

ENTER PICTURE NUMBER

26

PAUSE 000001
^P
        ARR NM =        26
        PIC NM =        26
        PERIM  =      24[illegible]
        AREA   =      353.[illegible]
        LENGTH =      806.0
        MAXWID =      240.5
```

```
[illegible]
 DAT

NO OF POINTS=   1167
NUMBER=      54

 ENTER PICTURE NUMBER

54

PAUSE 000001
^P
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        PIC.NM =       54
        PERIM. =   1883.0
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Figure 4. Maxima lengths and maxima widths superimposed on computer-generated images.

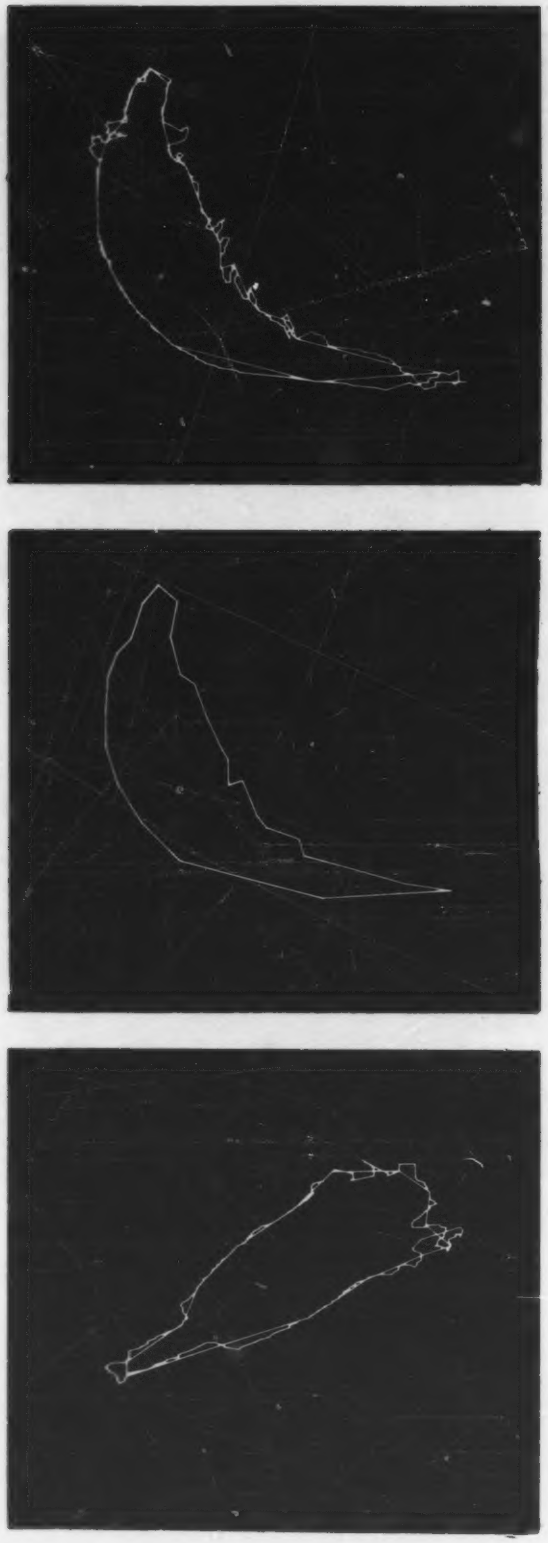

Fig. 5. Smoothed and unsmoothed computer generated images.

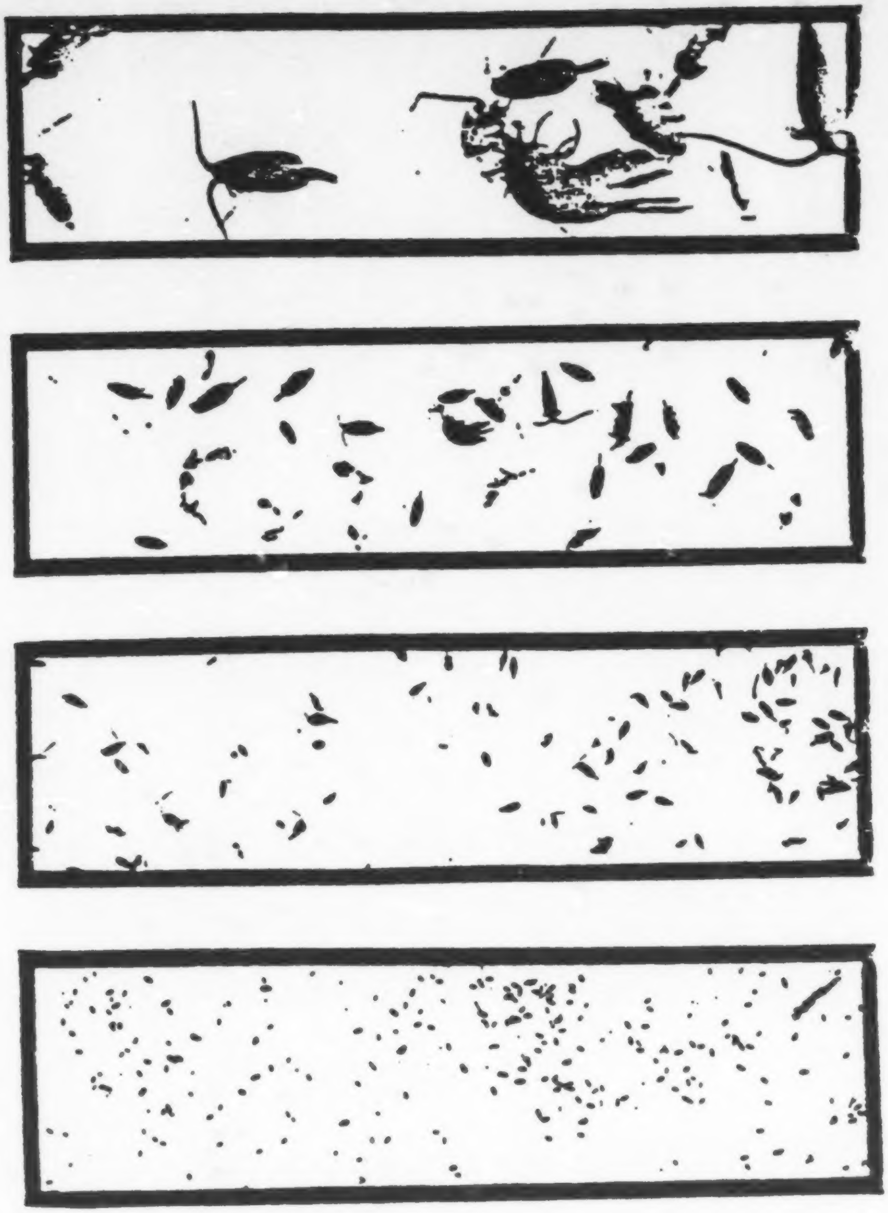

Fig. 6. Silhouette photographs of plankton.

```
S T A T I S T I C A L   A N A L Y S I S   S Y S T E M
                                                        18:00 WEDNESDAY, F
PLOT OF X2*X4    SYMBOL IS VALUE OF GRNUM
```

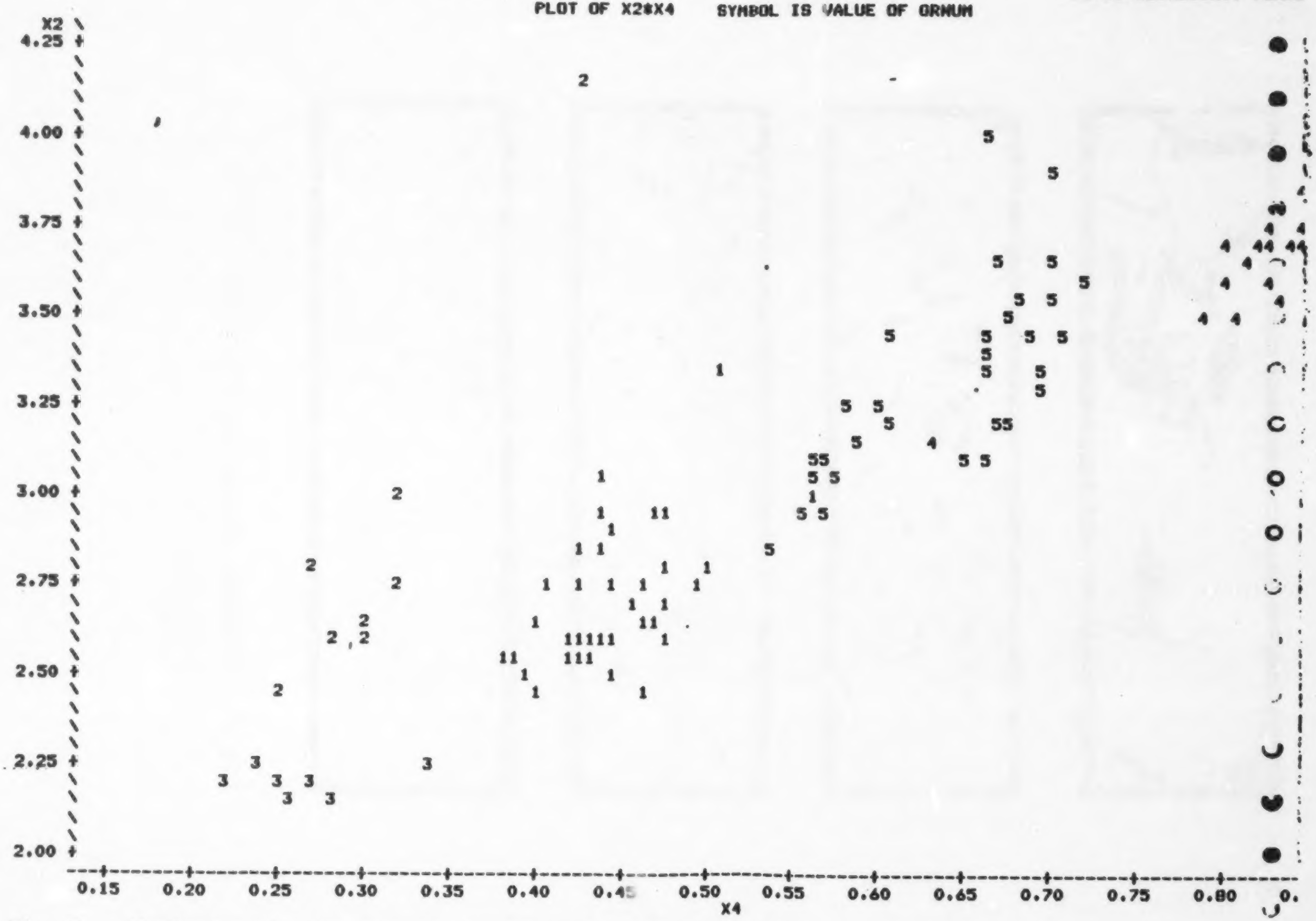

Fig. 7. Clustering of silhouettes in two-dimensional feature space.

```
S T A T I S T I C A L   A N A L Y S I S   S Y S T E M          16:47 THURSDAY, FEBRU

                   PLOT OF X2*X4    SYMBOL IS VALUE OF GRNUM
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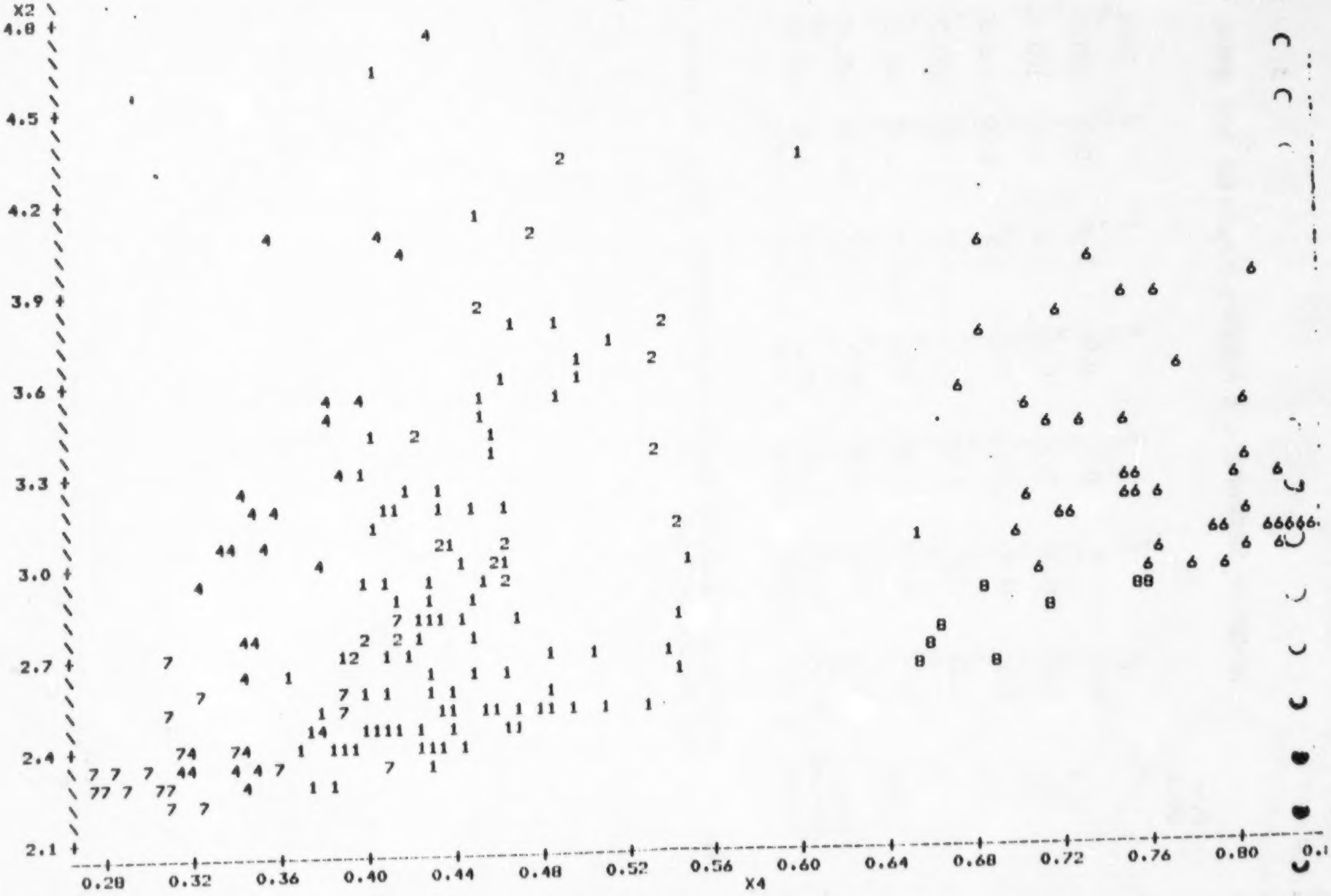

Fig. 8. Clustering of real plankton in two-dimensional feature space.

```
            NUMBER OF OBSERVATIONS & PERCENTS CLASSIFIED INTO GRNUM:

From
GRNUM
       1         1        2        4        6        7        8    TOTAL
                17        1        0        0        0        0       18
             94.44     5.56     0.00     0.00     0.00     0.00   100.00
       2         0        2        0        0        0        0        2
              0.00   100.00     0.00     0.00     0.00     0.00   100.00
       4         0        0        5        0        0        0        5
              0.00     0.00   100.00     0.00     0.00     0.00   100.00
       6         2        0        0        7        0        0        9
             22.22     0.00     0.00    77.78     0.00     0.00   100.00
       7         0        0        0        0        4        0        4
              0.00     0.00     0.00     0.00   100.00     0.00   100.00
       8         0        0        0        1        0        1        2
              0.00     0.00     0.00    50.00     0.00    50.00   100.00
Total           19        3        5        8        4        1       40
Percent      47.50     7.50    12.50    20.00    10.00     2.50   100.00
Priors      0.4350   0.0850   0.1150   0.2450   0.0850   0.0350
```

Fig. 9. Discriminant analysis results with training sample consisted of all 240 observations.

```
          NUMBER OF OBSERVATIONS AND PERCENTS CLASSIFIED INTO GRNUM:

From
GRNUM        1        2        4        6        7        8    Total
  1        103        2        0        0        0        0      105
         98.10     1.90     0.00     0.00     0.00     0.00   100.00
  2          0       19        0        0        0        0       19
          0.00   100.00     0.00     0.00     0.00     0.00   100.00
  4          0        0       27        0        1        0       28
          0.00     0.00    96.43     0.00     3.57     0.00   100.00
  6          0        0        0       58        0        0       58
          0.00     0.00     0.00   100.00     0.00     0.00   100.00
  7          1        0        0        0       20        0       21
          4.76     0.00     0.00     0.00    95.24     0.00   100.00
  8          0        0        0        3        0        6        9
          0.00     0.00     0.00    33.33     0.00    66.67   100.00
Total      104       21       27       61       21        6      240
Percent  43.33     8.75    11.25    25.42     8.75     2.50   100.00
Priors  0.4375   0.0792   0.1167   0.2417   0.0875   0.0375
```

Fig. 10. Discriminant analysis results with training sample consisted of 200 observations and a test set of 40 observations.

```
         NUMBER OF OBSERVATIONS AND PERCENTS CLASSIFIED INTO GRNUM:

From
GRNUM          1          2          4          6          7          8      Total
  1           17          1          0          0          0          0         18
           94.44       5.56       0.00       0.00       0.00       0.00     100.00
  2            0          2          0          0          0          0          2
            0.00     100.00       0.00       0.00       0.00       0.00     100.00
  4            0          0          4          0          1          0          5
            0.00       0.00      80.00       0.00      20.00       0.00     100.00
  6            1          0          0          8          0          0          9
           11.11       0.00       0.00      88.89       0.00       0.00     100.00
  7            0          0          0          0          4          0          4
            0.00       0.00       0.00       0.00     100.00       0.00     100.00
  8            0          0          0          2          0          0          2
            0.00       0.00       0.00     100.00       0.00       0.00     100.00
Total         18          3          4         10          5          0         40
Percent    45.00       7.50      10.00      25.00      12.50       0.00     100.00
Priors    0.4350     0.0850     0.1150     0.2450     0.0850     0.0350
```

Fig. 11. Discriminant analysis results of smoothed plankton.

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6	
	Miscl.	%	Miscl.	%	Miscl.	%	Miscl.	%	Miscl.	%	Miscl.	%
Original Training	3	1.50	4	2.00	7	3.50	4	2.00	4	2.00	3	1.50
Original Test	4	10.00	5	12.50	3	7.50	8	20.00	8	20.00	5	12.50
Smoothed Training	11	5.50	10	5.00	11	5.50	13	5.50	11	5.50	11	5.50
Smoothed Test	5	12.50	9	22.50	3	7.50	8	20.00	10	25.00	9	22.50

	Total (Mean)	
	Miscl.	%
Original Training	4.17	2.09%
Original Test	5.50	13.75%
Smoothed Training	11.17	5.58%
Smoothed Test	7.32	18.33%

Fig. 12. Mean misclassification error.

```
FREQUENCY    0    2    6    3    5    2    2    1    5    2
----------+----+----+----+----+----+----+----+----+----+----+---
    6                  5
    5                  5         5                   5
    4                  5         5                   5
    3                  5    5    5                   5
    2             5    5    5    5    5    5         5    5
    1             5    5    5    5    5    5    5    5    5
----------+----+----+----+----+----+----+----+----+----+----+---
         0.500     1.100     1.700     2.300     2.900     3.500
              0.800     1.400     2.000     2.600     3.200
```

```
FREQUENCY    2    2    4    4    6    2    2    1    4    1
----------+----+----+----+----+----+----+----+----+----+----+---
    6                            6
    5                            6
    4                  6    6    6                   6
    3                  6    6    6                   6
    2        6    6    6    6    6    6    6         6
    1        6    6    6    6    6    6    6    6    6    6
----------+----+----+----+----+----+----+----+----+----+----+---
         2.000     3.600     5.200     6.800     8.400    10.000
              2.800     4.400     6.000     7.600     9.200
```

Fig. 13. a) Distribution of (square root of area)/(maximum width).
b) Distribution of (maximum length)/(maximum width).

```
FREQUENCY   10   23   14    4    4    1    0    1
----------+----+----+----+----+----+----+----+----+----+----+---
   23             3
   22             3
   21             3
   20             3
   19             3
   18             3
   17             3
   16             3
   15             3
   14             3    3
   13             3    3
   12             3    3
   11             3    3
   10        3    3    3
    9        3    3    3
    8        3    3    3
    7        3    3    3
    6        3    3    3
    5        3    3    3
    4        3    3    3    3    3
    3        3    3    3    3    3
    2        3    3    3    3    3
    1        3    3    3    3    3    3         3
----------+----+----+----+----+----+----+----+----+----+----+---
         0.155     0.165     0.175     0.185     0.195
              0.160     0.170     0.180     0.190
```

Fig. 14. Distribution of first invariant moment function.

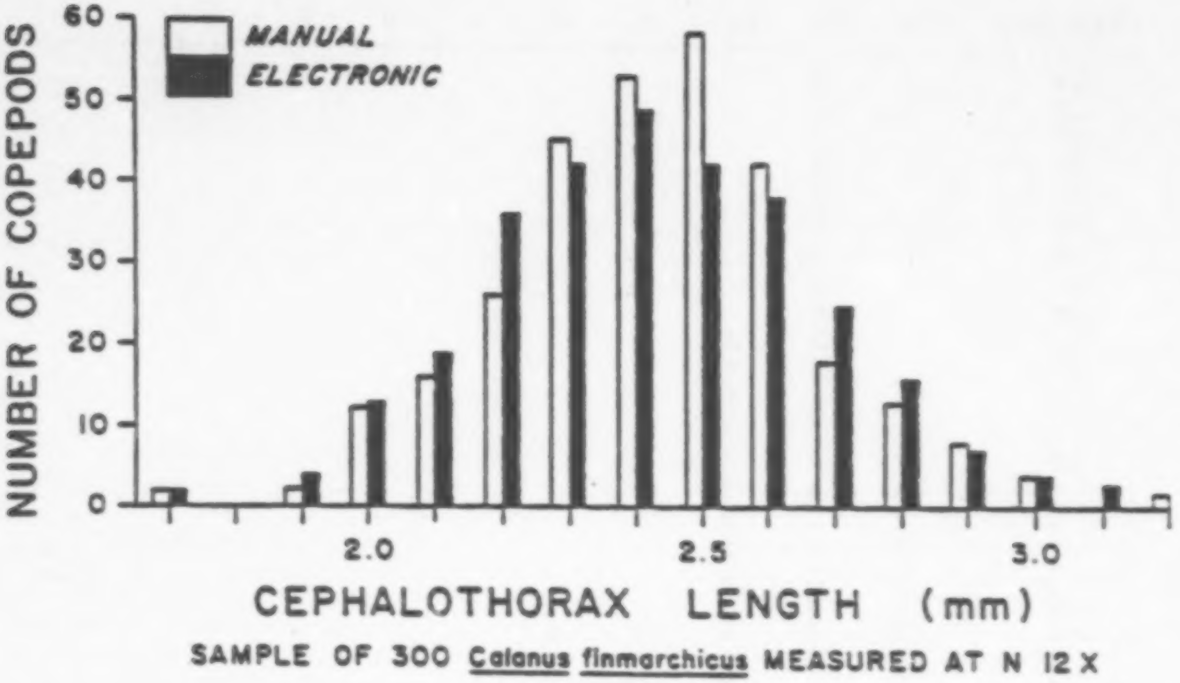

Fig. 15. Error distribution of length measurement.

COMPUTER INTERFACED SYSTEMS FOR BIOLOGICAL MONITORING OF WATER QUALITY

by

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INTRODUCTION

Effectiveness of pollution control historically has been assessed by chemical/physical parameters. Yet the goal of pollution control is to protect living things. However, no equation exists that permits the precise conversion of the effects of chemical/physical alteration of the environment upon living things. No probe devised by man will measure toxicity or will measure the aggregate effect of multiple physical/chemical alterations upon aquatic biota. However, alterations of the physical/chemical quality of a receiving system rarely occur singly. An ordinary run-of-the-mill industrial waste discharge may typically contain 30 or more constituents. Furthermore, the concentrations of these constituents frequently vary markedly due to production and process changes that are cyclical. The problem is compounded further by the presence of many industrial and municipal discharges in an aquatic ecosystem and the fact that the natural environment itself rarely remains constant, adding yet another set of changing conditions.

Abundant evidence exists that water hardness, temperature, dissolved oxygen concentration, pH, and a variety of other charcteristics may markedly alter the response of an organism to a chemical. The use of organisms as environmental condition sensors has been vastly underutilized by those charged with the responsibility for environmental quality control. As a consequence of the failure to use living organisms as sensors, regulatory and waste treatment measures designed to protect the organisms are rarely as effective as they otherwise would be. It is worth noting, however, that in the absence of chemical/physical information the factors which cause the biological response will very likely be missed. It is, therefore, necessary to couple biological/chemical/physical data for effective environmental protection and quality control.

No quality control system can operate effectively without a rapid feedback of information about the quality of the system being controlled. This monitoring of conditions is necessary in order to take immediate corrective measures when the quality falls below acceptable levels. This simple and seemingly obvious fact has been ignored almost entirely where environmental quality control practices are concerned. One or two simple measurements such as BOD, pH, or temperature are taken occasionally, but practically never is the condition of the biota in the receiving system measured continuously as would be the case in almost any other quality control system. The reasons for this (other than the failure of the biologists to develop methods which is no longer a valid reason for not engaging in biological monitoring) are (a) the cost of continual biological monitoring and (b) the long time customarily required for examination of biological material, and the analysis and reporting of the condition.

In 1966, it was quite clear that biological monitoring as a routine and regular practice would not be widely used even if federal and state regulatory agencies proposed it until such monitoring became cost effective and sufficiently rapid to enable corrective measures to be taken promptly. One of the earlier papers on this topic was Cairns et al. (1971). This paper described an "inplant" early warning system designed to detect deleterious materials before they left the plant site and entered the receiving system, and an "instream" method which permitted relatively rapid assessment of community condition of the biota in the receiving system. The inplant system involved a single species. The instream system involved a community of species. The inplant system was automated; the instream system was at that time not automated. Subsequently, it seemed desirable to provide a demonstration that most of the commonly used parameters to assess deleterious effects of pollution on aquatic biota could be automated with the use of simple electronic equipment. A number of categories was chosen for demonstration, that is, (a) physiological condition as measured by respiratory (ventilatory) response, (b) changes in behavior, (c) changes in community structure as measured by diversity indices and indicator species ratios, and (d) changes in response to a rapidly changing wastewater in which both the quantity and quality fluctuated as it does in the "real world." This system would be totally unlike the steady state exposures common to practically all toxicity tests.

EARLY WARNING SYSTEM

An automated early warning system has been described in detail elsewhere (e.g., Cairns et al., 1973; Cairns, 1972; Cairns, 1975a,b) and will not be discussed extensively here. The system has also had site specific testing (e.g., van der Schalie et al., 1979; Gruber et al., 1978). Both salt and freshwater fish species may be used as sensors (Cairns et al., 1980). The test chamber is shown in figure 1 and the wastewater testing schematic is shown in figure 2. The diurnal patterns and distress response are depicted in figure 3 and the decision diagram in figure 4. A comparison of this system with others has been made (Cairns and Gruber, 1980).

AUTOMATED IDENTIFICATION

The automated identification unit is a pattern recognition system not much different operationally from the FBI fingerprint matching system. Detailed descriptions of the system may be found in Cairns et al. (1972 and 1977) and a check of its accuracy is presented in Cairns et al. (1979). Figure 5 shows how the structural pattern is recorded and figure 6 shows 12 optical spatial filters in a 2" x 2" glass photographic plate. Figure 7 shows a schematic of the system and figure 8 shows the correlation dots which appear when a pattern is "recognized." Figures 9 and 10 show how the information generated could be used to assess pollution. Each count would require about 40 hours of skilled technician time using traditional methods, but less than an hour would be required with automation.

AUTOMATION OF BEHAVIOR ANALYSIS

The analysis of fish pereference and avoidance of certain physical and chemical conditions is important when estimating the effect of an industrial waste mixing zone in a river or lake. Upstream migration could be blocked or impaired if fish were repelled by the material. Conversely, the fish could be killed if a waste attracted them into lethal concentrations of chemicals. The system was not designed to duplicate natural conditions but rather to get a preliminary estimate of preference, avoidance, and induction of aberrant behavior. Figure 11 (from Lubinski et al., 1977) provides a schematic of the system. Figure 12 illustrates one type of evidence provided. Additional information may be obtained from Lubinski et al. (1978).

SIMULATION OF VARIABLE EFFLUENT DISCHARGES

Practically every labortatory toxicity test is designed to give a constant dose of a particular chemical to a particular test organism. While steady state concentration is often difficult to achieve, variability in toxicant concentration occurs accidentally rather than as a matter of intent to do so. The Mount-Brungs serial diluters and other such devices were designed to enhance the probability of providing constant concentrations of test materials which might not easily remain at a constant concentration in nonrenewable batch testing systems. In the "real world," however, industrial discharges are rarely constant in quality, and often not even in quantity, for a substantial period of time. It would be astonishing if discharges remained constant for the most commonly used toxicity testing exposure period of 96 hours. Therefore, laboratory tests with constant conditions are not particularly reflective of the types of dosing or exposure experienced by organisms in the "real world."

The scientific method encourages keeping everything constant in an experiment except one variable (the one of particular interest in the study). Thus, the kind of constancy just described for laboratory testing is ideal if one wishes to get manuscripts accepted for publication in scholarly journals but does provide some difficulty when attempting to determine the relationship between this type of exposure and the exposure actually experienced by aquatic organisms. In addition to the reason just described, there are three additional reasons why such experiments are so rare, that is, (a) it is difficult to control delivery of the test material so that the concentration is reasonably constant, let alone controlling one deliberately designed to be variable; (b) it is difficult to preprogram such delivery in such a way that one can be assured that it actually will occur; and (c) it is difficult to record response of test organisms to highly variable conditions unless one has a continuously recording system. The system now under development for two years at this institution addresses all the aspects just mentioned.

Although it is worth emphasizing that this is still an experimental system subject to the usual failures and uncertainties of any research activity, the evidence generated thus far indicates that the system may very well be suitable for testing the effects of variable waste loadings. A more detailed description of the apparatus and some of the test results may be found in Cairns and Thompson (in press), and a description of the statistical analysis used may be found in Thompson et al. (in press).

Figure 13 gives a schematic of the system used (from Cairns and Thompson, in press). Figure 14 shows a constant exposure and the variable response of the fish to it and figure 15 shows a pulsed exposure and the response of the fish to it. Note the recovery following the peak response.

CONCLUSION

Four types of computer interfaced systems designed to acquire biological response to pollutants are described. One (the early warning system) has been in development since 1966 and has been tested for over a year at one industrial site and for several years at another. The automated identification unit has been under development since 1970 and should shortly be ready for practical use if this type of information in this quantity is needed. The various forms of the behavior system have been under development since 1966 and have been tested in several practical situations. The unit designed to simulate fluctuating effluent discharges has been in development since 1978 and appears promising, although much additional work is needed before it can be routinely used for applied problem solving.

The illustrations provided show that certain types of biological pollution assessment can be automated, and the information can be directly coupled with chemical/physical information. The minicomputer systems may be used not only to generate data but also for storage and analysis. These procedures substantially increase the amount of data that can be handled, reduce the time needed to analyze data, and vastly improve the opportunity for statistical evaluation of the data. Data storage and retrieval are also markably enhanced. Additionally, through the use of leased telephone lines, data can be transmitted to larger computers with more sophisticated capabilities should these be advantageous.

If biological assessment is to enjoy widespread use, the old fashioned labor intensive measurements must be replaced in part, especially where routine measurements are concerned, by the more cost effective and time efficient computer interfaced systems. Skills of highly trained persons would be better used since these persons would be free of routine determinations and therefore able to take fuller advantage of their analytical skills. Additionally, a rapid response time permits equally rapid corrective action and, more important, enables one to make closer correlations between alterations of chemical/physical conditions and the biological responses they elicit. Since the U.S. Environmental Protection Agency and presumably other federal agencies charged with environment responsibilties have a vastly increased work load (Carter, 1980) without a concomitant increase in funds and personnel, it would appear advantageous to automate whenever possible, particularly since not only data generation, but data analysis, storage, retrieval, and other activities now labor intensive can be handled by "shelf" electronics equipment.

ACKNOWLEDGMENT

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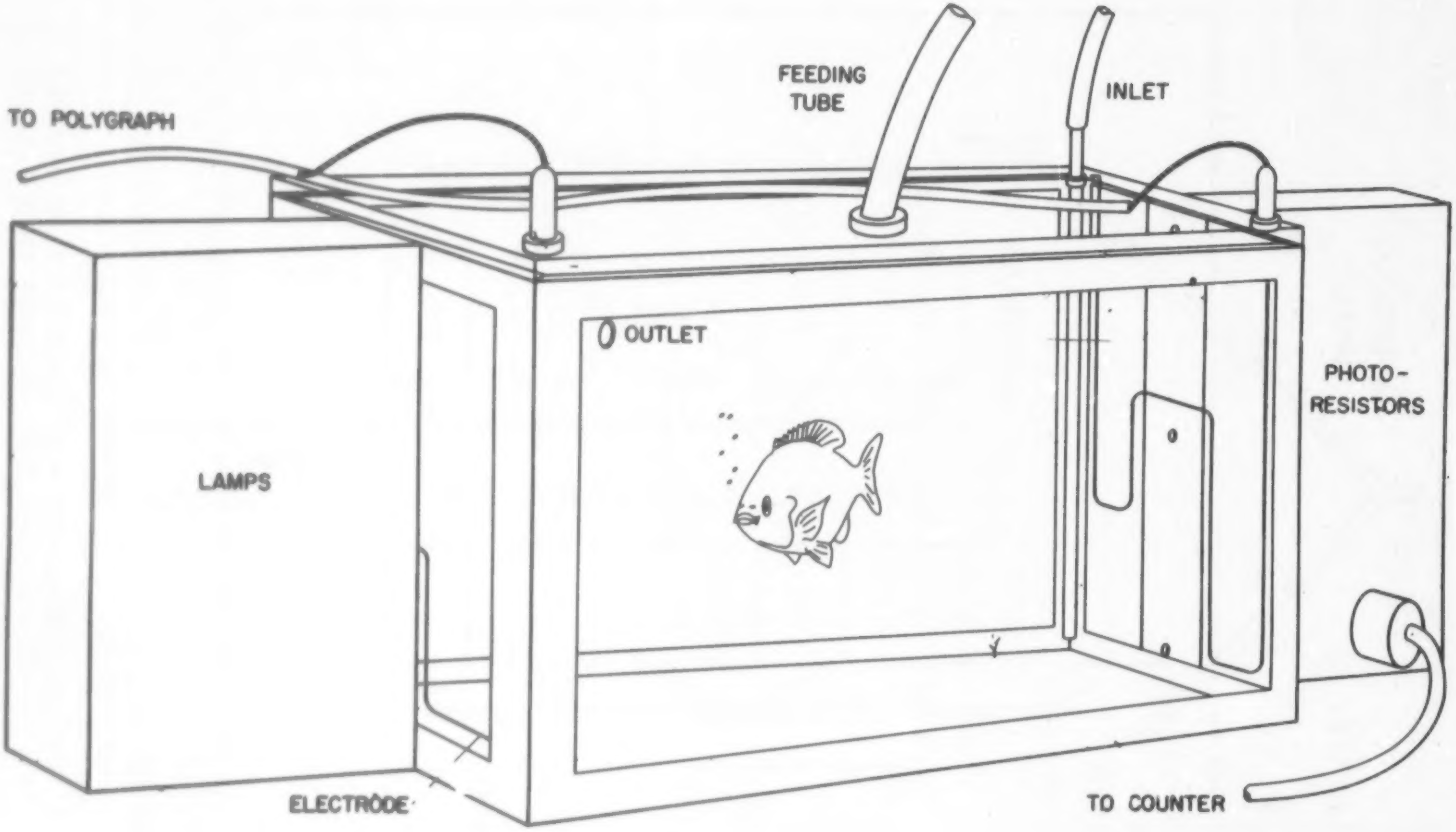
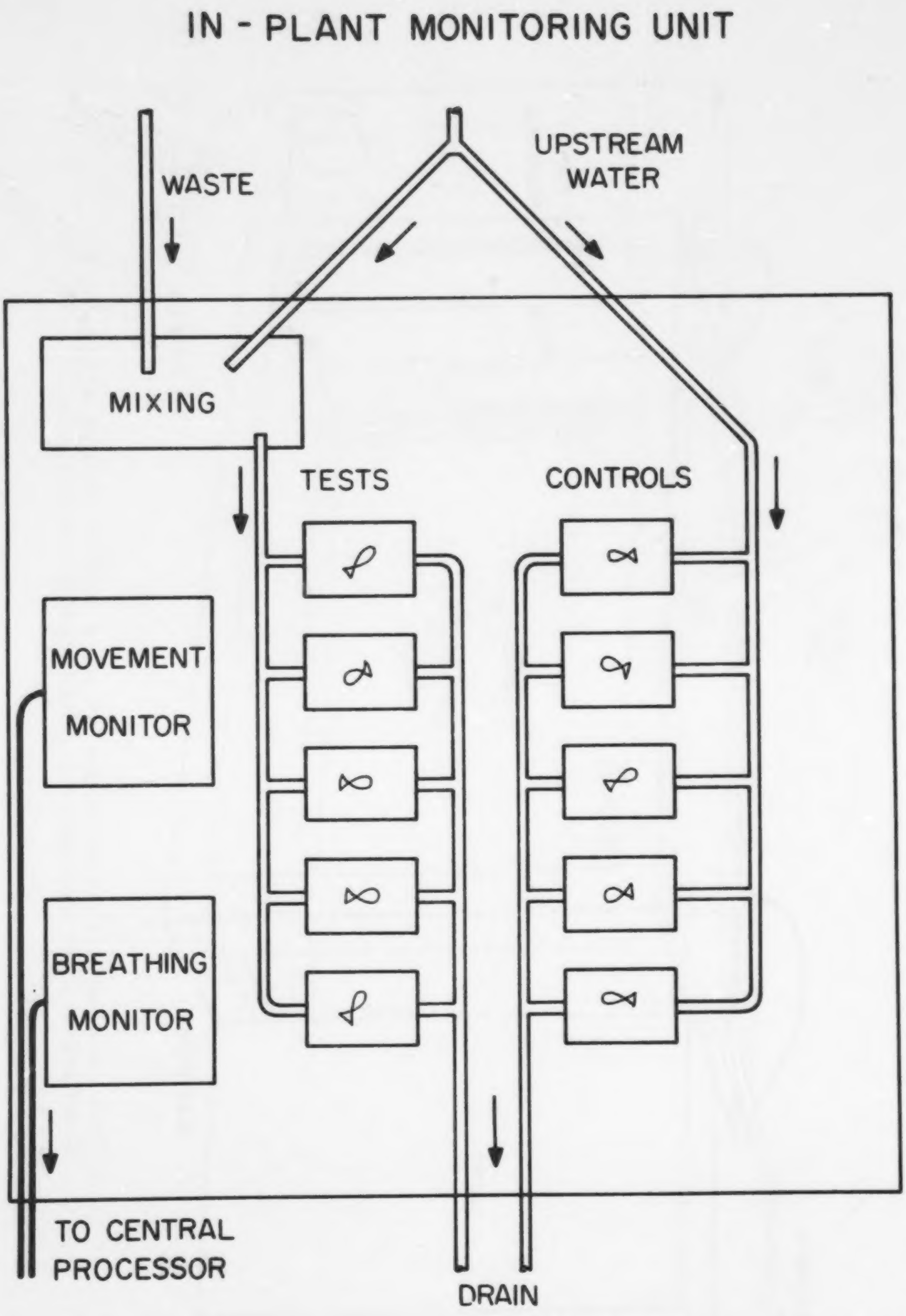
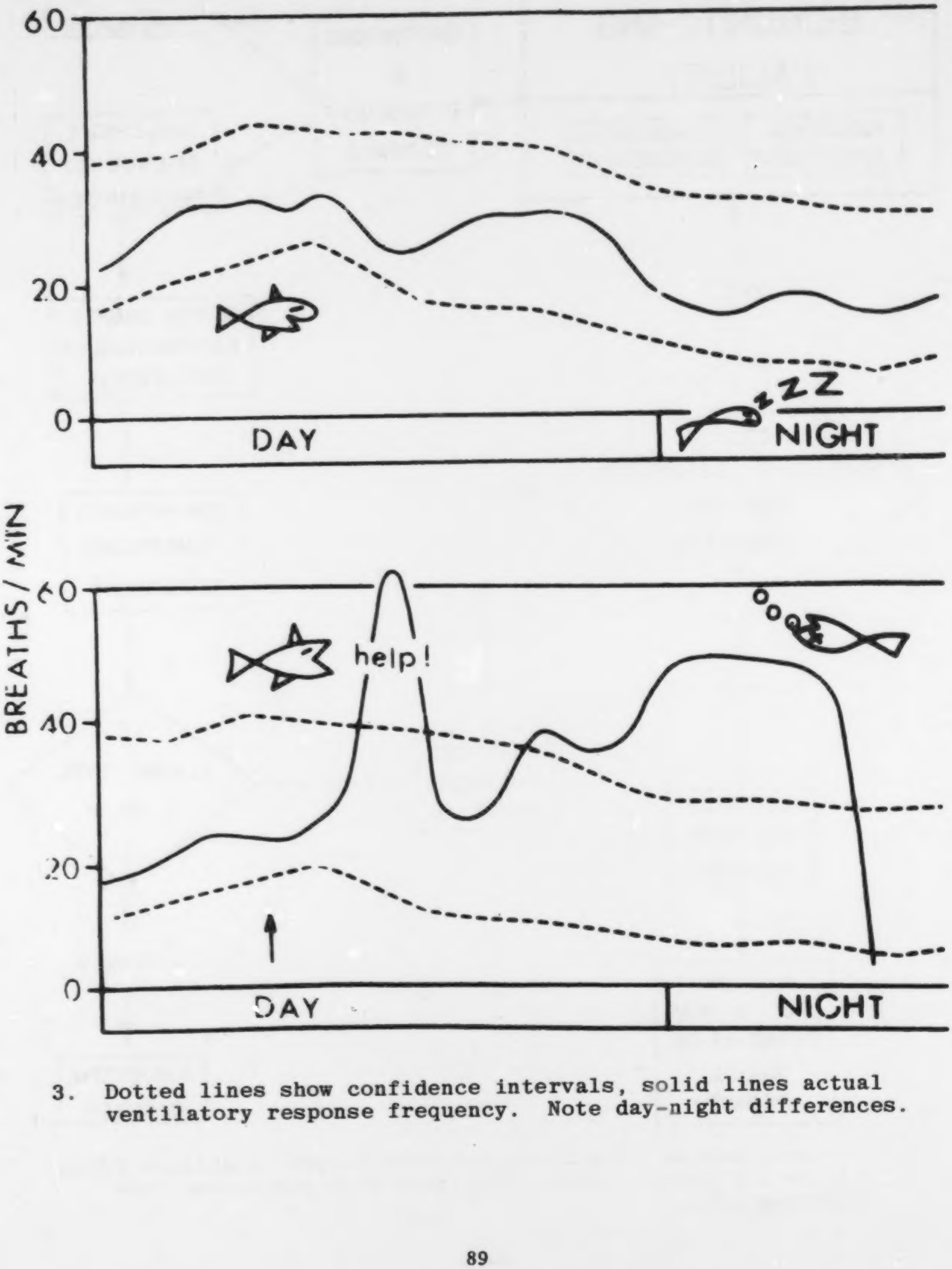

TO POLYGRAPH
FEEDING TUBE
INLET
OUTLET
PHOTO-RESISTORS
LAMPS
ELECTRODE
TO COUNTER

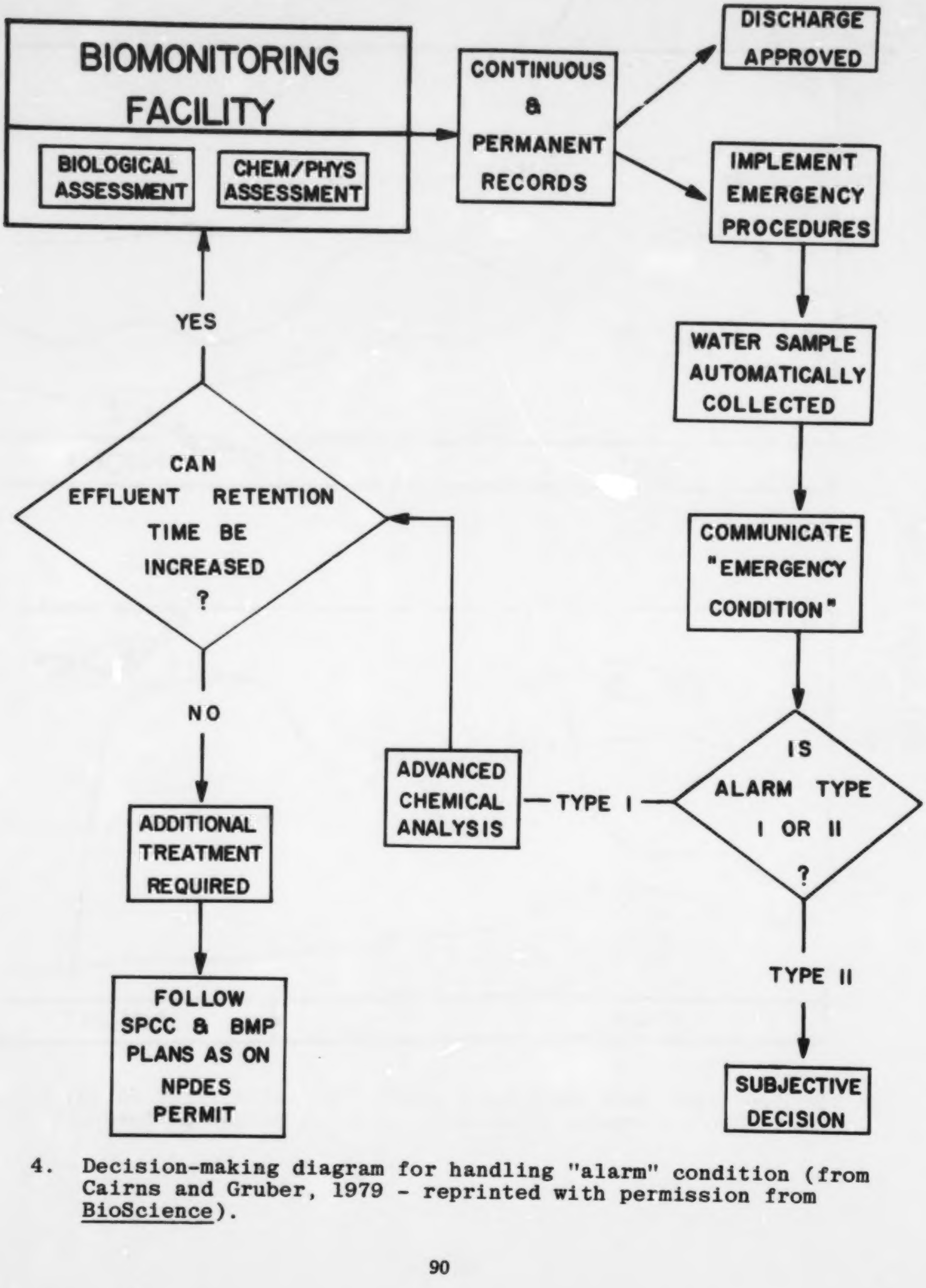
1. Test chamber with electrodes at each end to record electrical discharges from ventilatory ("breathing") movements.


IN - PLANT MONITORING UNIT
WASTE
UPSTREAM WATER
MIXING
TESTS
CONTROLS
MOVEMENT MONITOR
BREATHING MONITOR
TO CENTRAL PROCESSOR
DRAIN

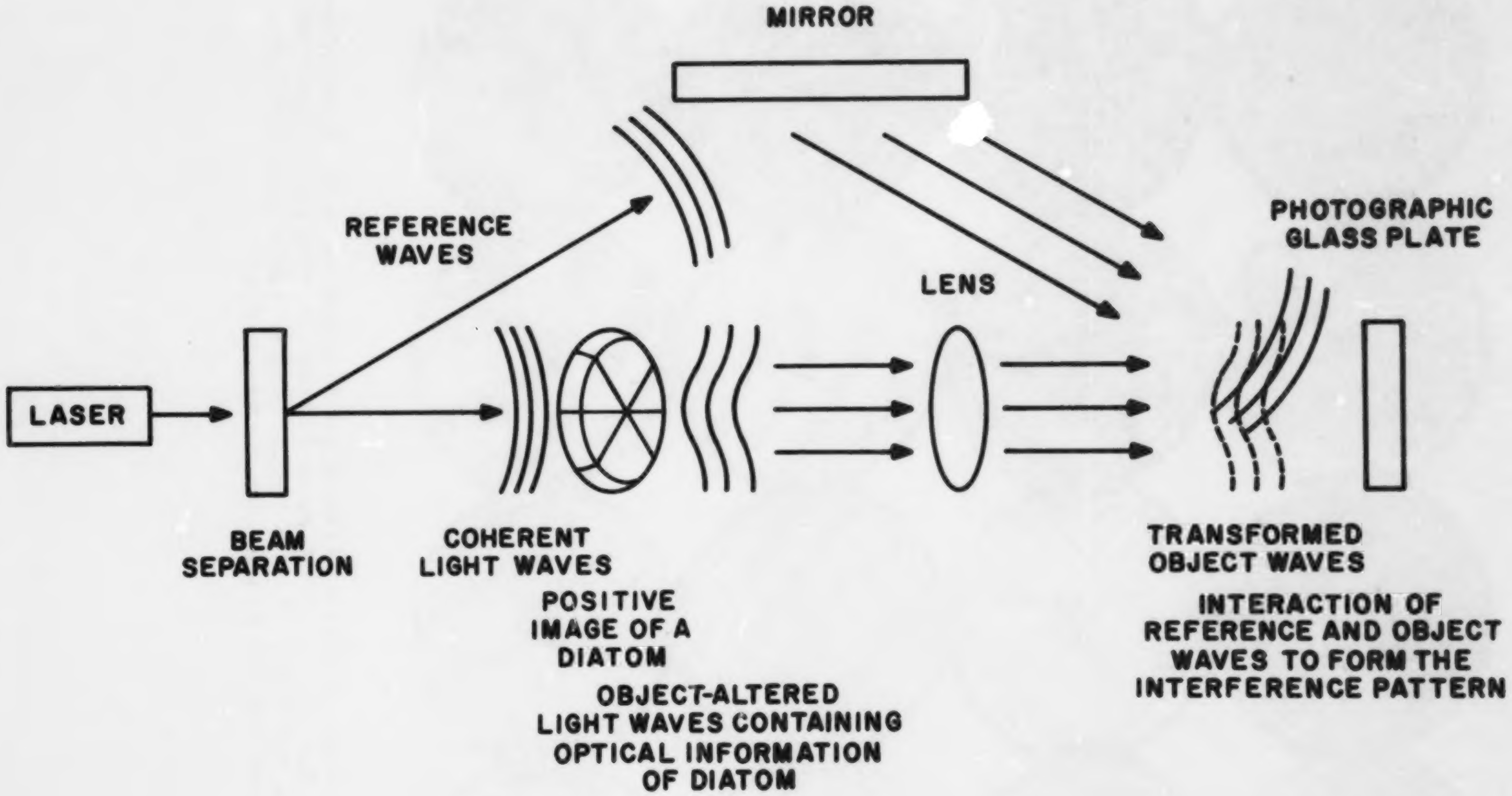
2. An inplant monitoring system with control fish exposed to dilution water only and test fish exposed to a mixture of wastewater and dilution water.



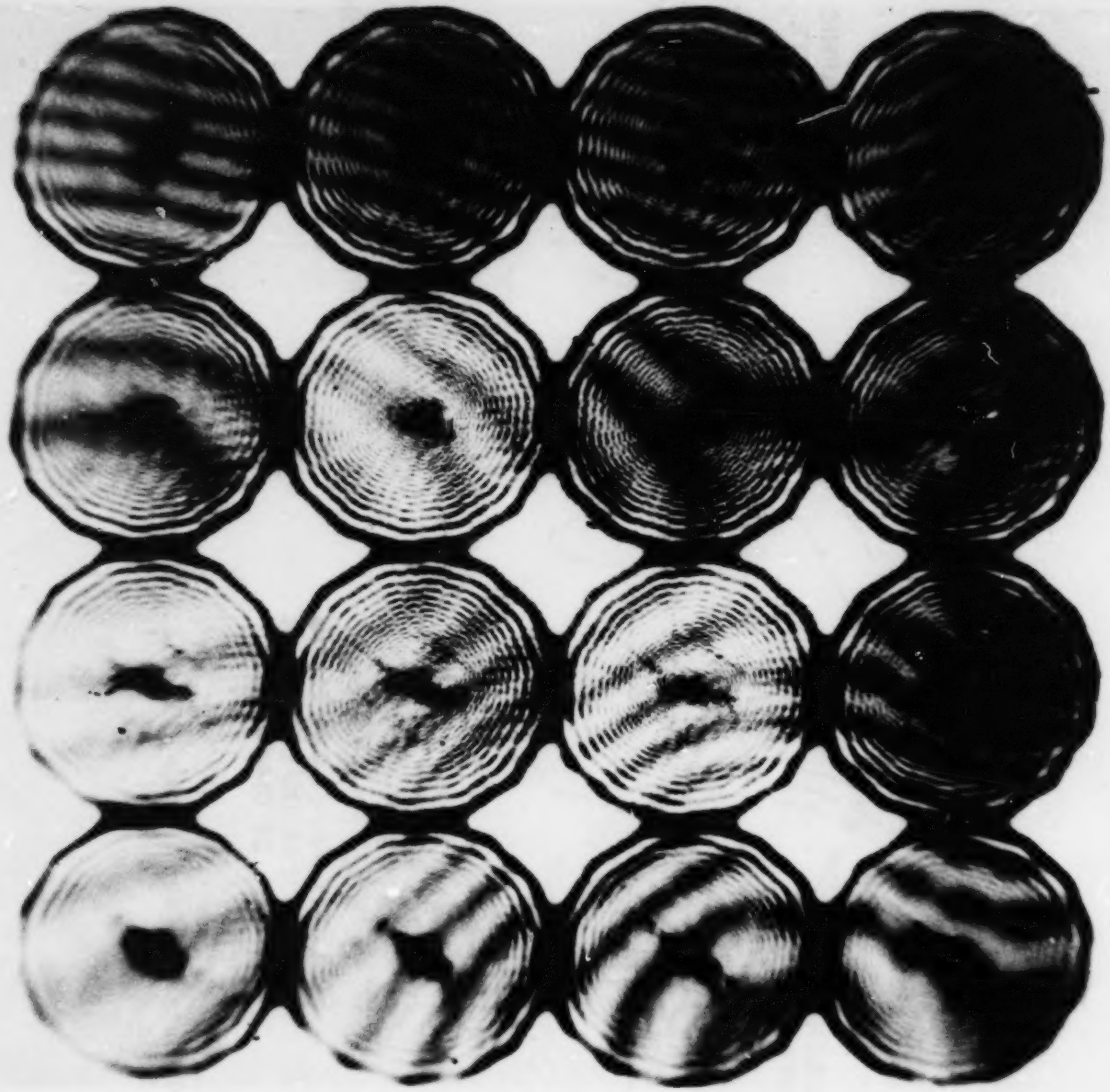
3. Dotted lines show confidence intervals, solid lines actual ventilatory response frequency. Note day-night differences.


BIOMONITORING FACILITY
BIOLOGICAL ASSESSMENT
CHEM/PHYS ASSESSMENT
CONTINUOUS & PERMANENT RECORDS
DISCHARGE APPROVED
IMPLEMENT EMERGENCY PROCEDURES
WATER SAMPLE AUTOMATICALLY COLLECTED
COMMUNICATE "EMERGENCY CONDITION"
IS ALARM TYPE I OR II ?
TYPE I
ADVANCED CHEMICAL ANALYSIS
TYPE II
SUBJECTIVE DECISION
CAN EFFLUENT RETENTION TIME BE INCREASED ?
YES
NO
ADDITIONAL TREATMENT REQUIRED
FOLLOW SPCC & BMP PLANS AS ON NPDES PERMIT

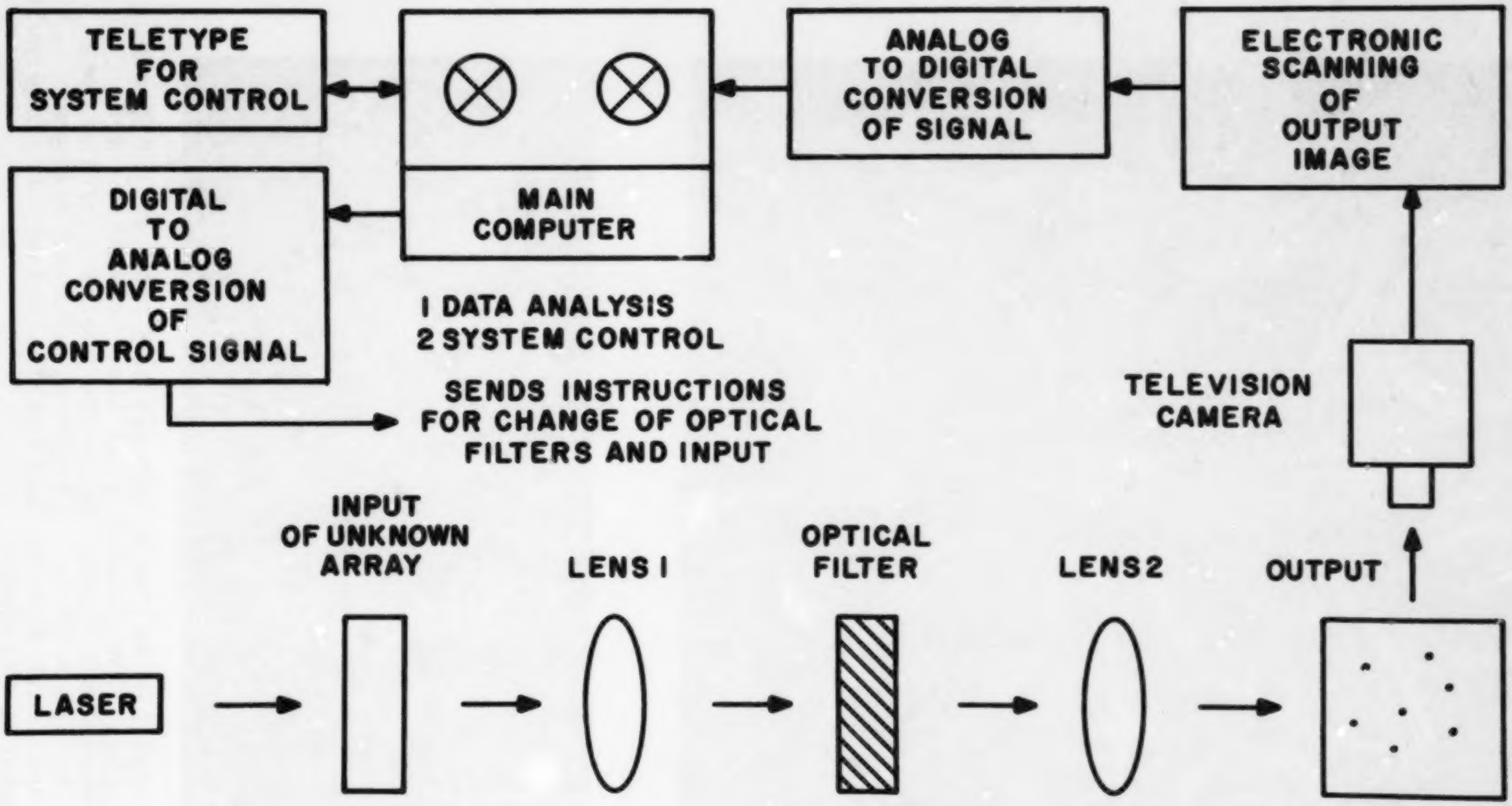
4. Decision-making diagram for handling "alarm" condition (from Cairns and Gruber, 1979 - reprinted with permission from BioScience).


MIRROR
REFERENCE WAVES
PHOTOGRAPHIC GLASS PLATE
LENS
LASER
BEAM SEPARATION
COHERENT LIGHT WAVES
POSITIVE IMAGE OF A DIATOM
OBJECT-ALTERED LIGHT WAVES CONTAINING OPTICAL INFORMATION OF DIATOM
TRANSFORMED OBJECT WAVES
INTERACTION OF REFERENCE AND OBJECT WAVES TO FORM THE INTERFERENCE PATTERN

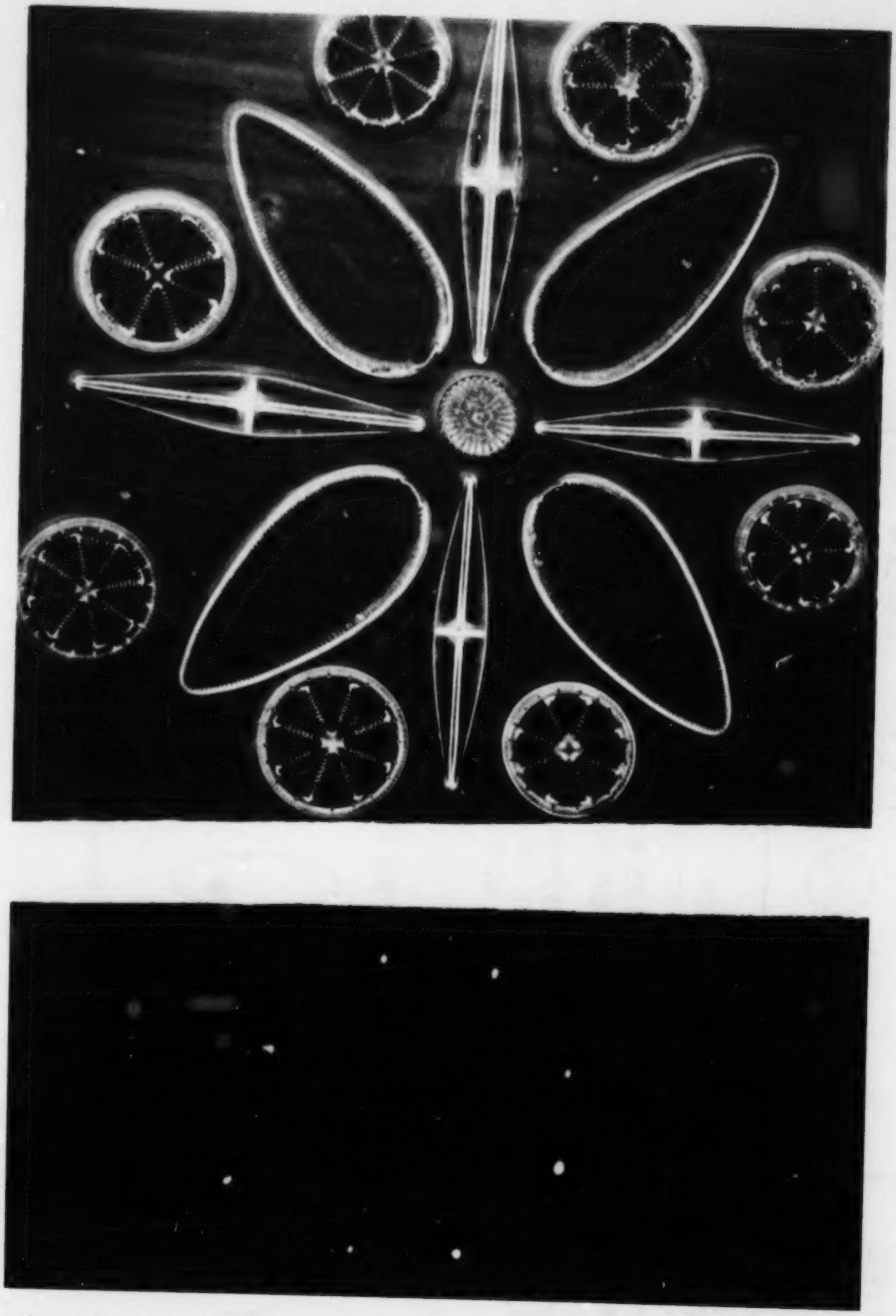
5. The preparation of an optical spatial filter for pattern recognition.



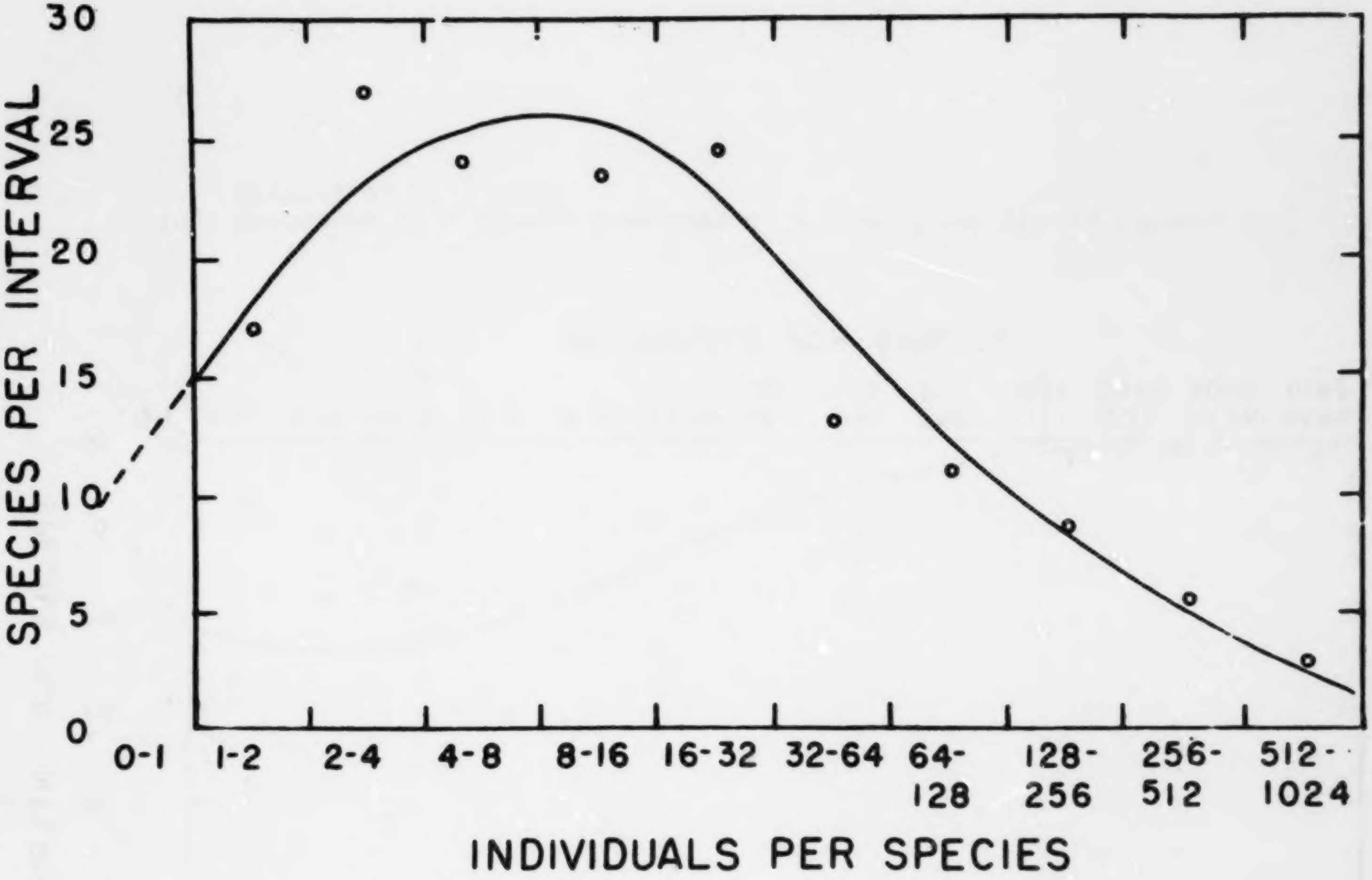
6. Twelve optical spatial filters positioned on a 2" X 2" glass plate. Since the size of each filter is generally inversely related to the size of the input image, approximately 100 such filters can be placed on a single plate.


TELETYPE FOR SYSTEM CONTROL
MAIN COMPUTER
ANALOG TO DIGITAL CONVERSION OF SIGNAL
ELECTRONIC SCANNING OF OUTPUT IMAGE
DIGITAL TO ANALOG CONVERSION OF CONTROL SIGNAL
1 DATA ANALYSIS
2 SYSTEM CONTROL
SENDS INSTRUCTIONS FOR CHANGE OF OPTICAL FILTERS AND INPUT
TELEVISION CAMERA
LASER
INPUT OF UNKNOWN ARRAY
LENS 1
OPTICAL FILTER
LENS 2
OUTPUT

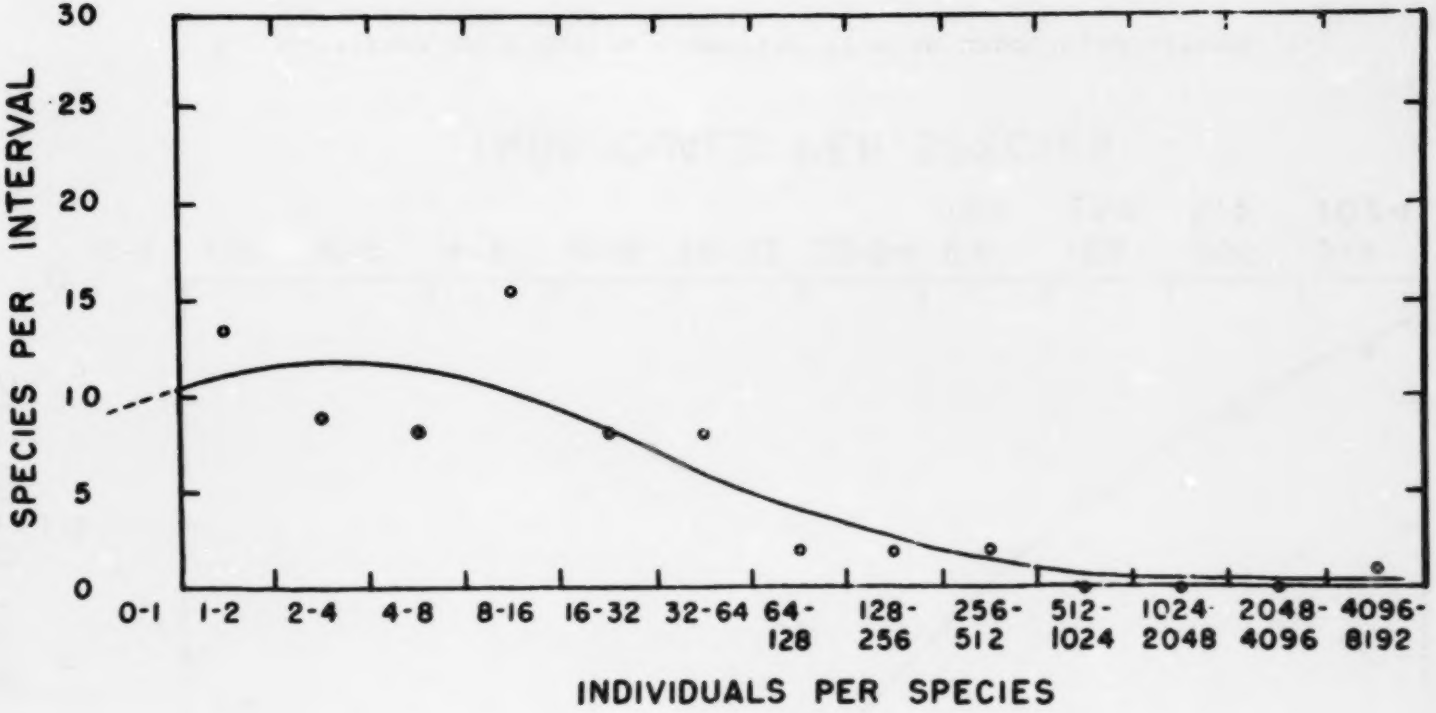
7. General schematic.



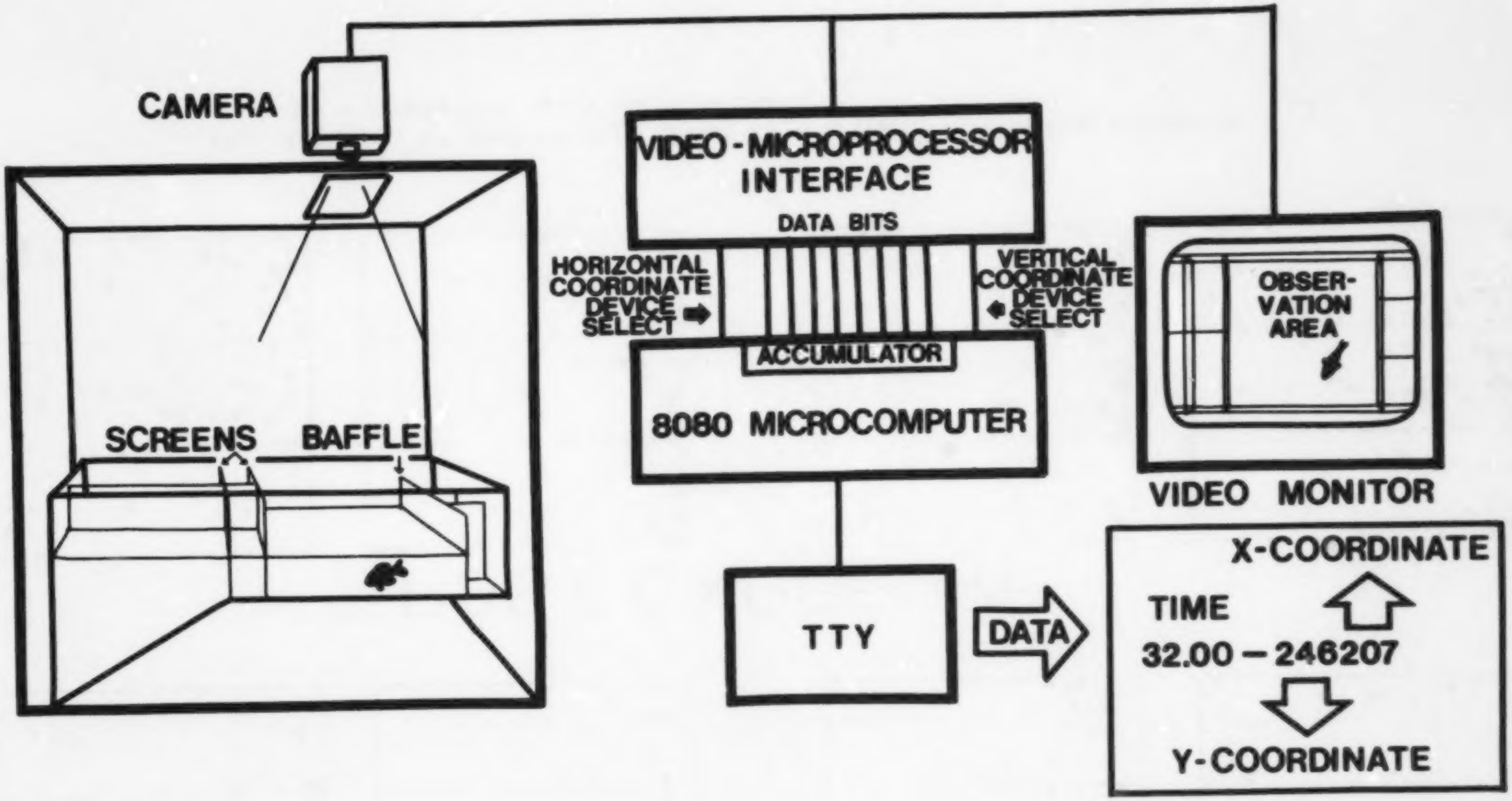
8. Input and output planes for a correlation test of the diatom *Heliopelta metii*. The intensity of the correlation dots indicate an excellent match. Note the position of the dots relative to the input array. (Slide of arrayed diatoms courtesy of Drs. Ruth Patrick and Charles Reiner, Philadelphia Academy of Natural Sciences).



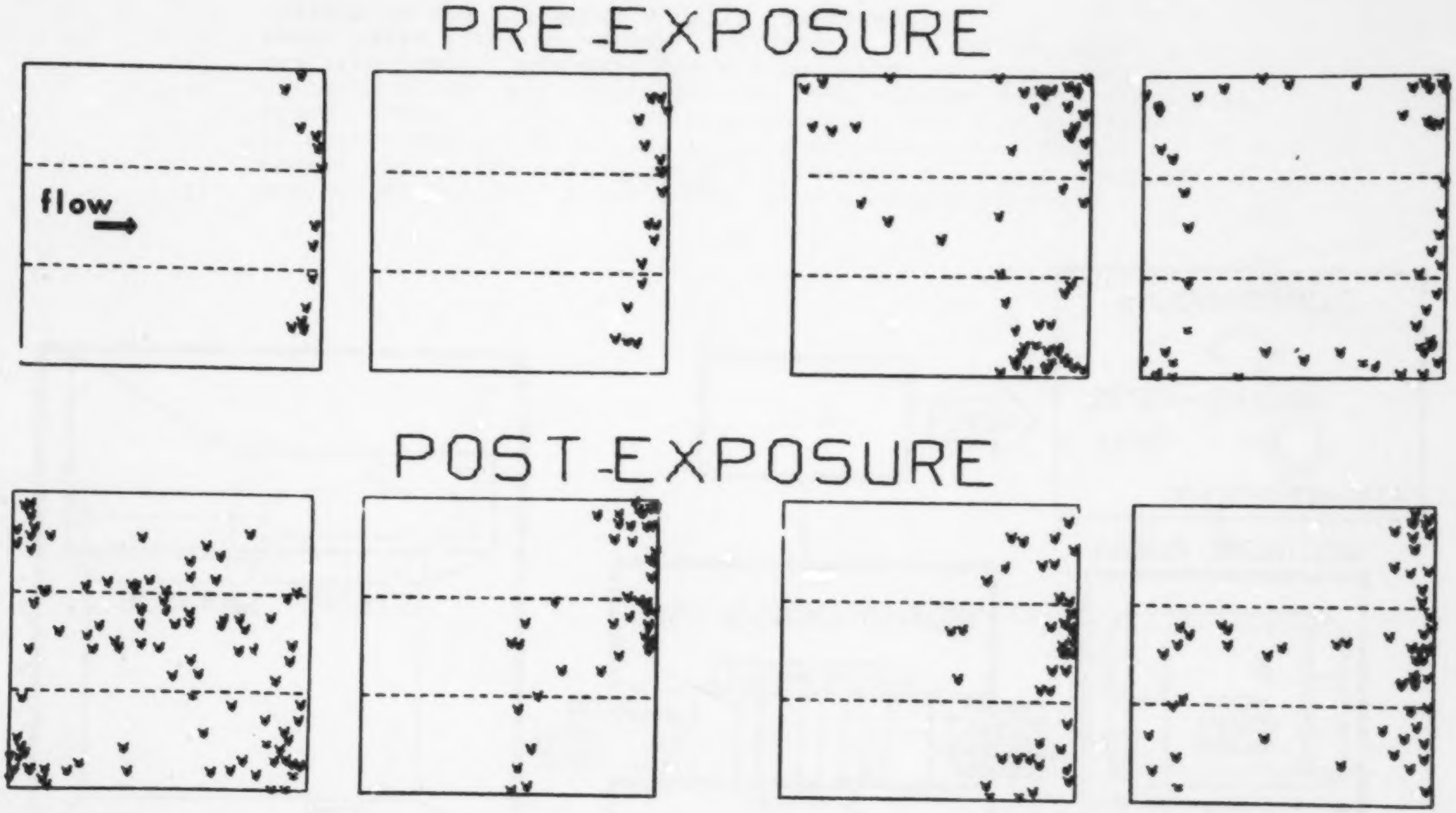
9. Structure of a diatom community from an unpolluted stream (from Patrick et al., 1954).



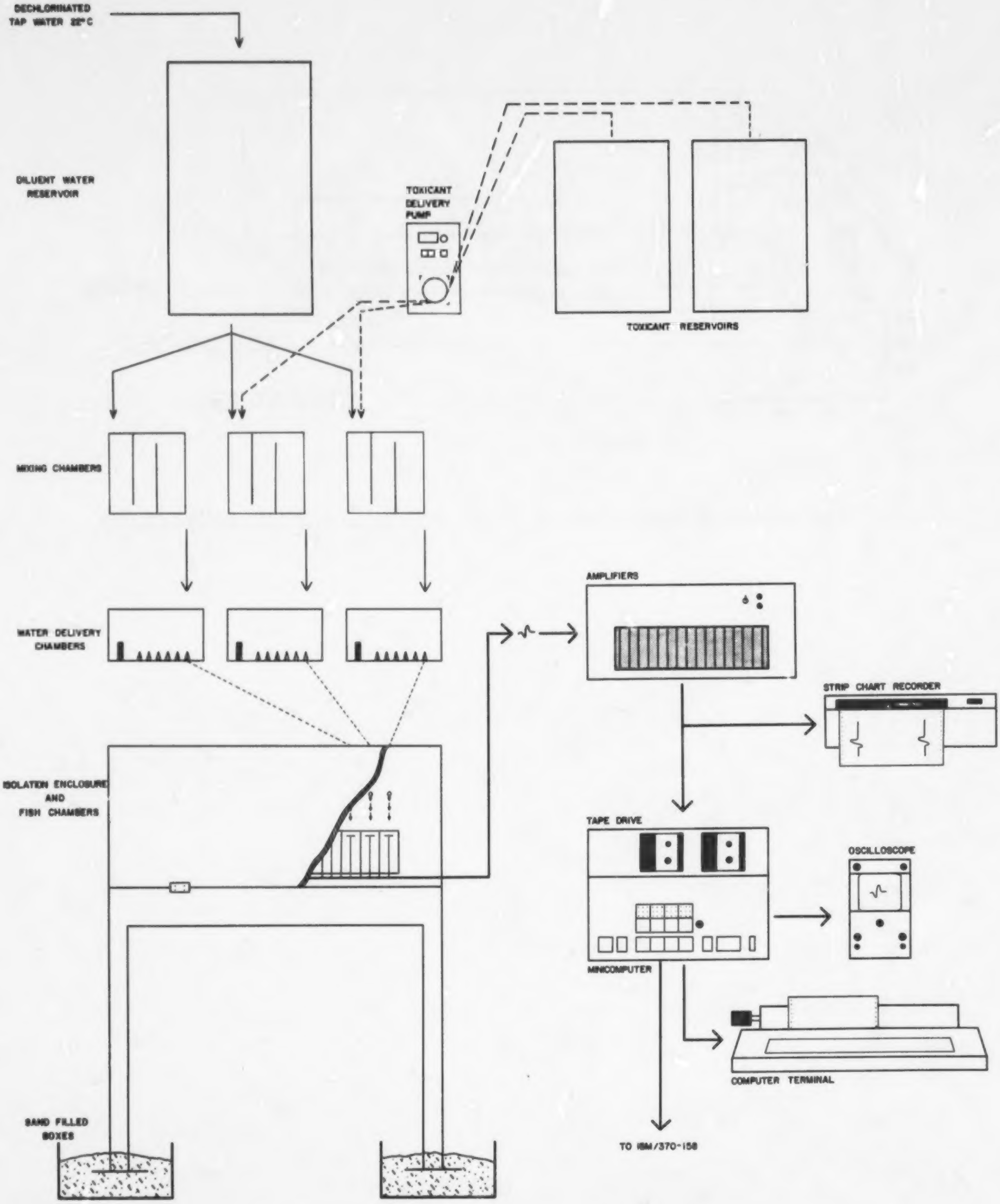
10. Structure of a diatom community from a polluted stream (from Patrick et al., 1954).


CAMERA
SCREENS
BAFFLE
VIDEO - MICROPROCESSOR INTERFACE
DATA BITS
HORIZONTAL COORDINATE DEVICE SELECT
VERTICAL COORDINATE DEVICE SELECT
ACCUMULATOR
8080 MICROCOMPUTER
OBSERVATION AREA
VIDEO MONITOR
TTY
DATA
X-COORDINATE
TIME
32.00 — 246207
Y-COORDINATE

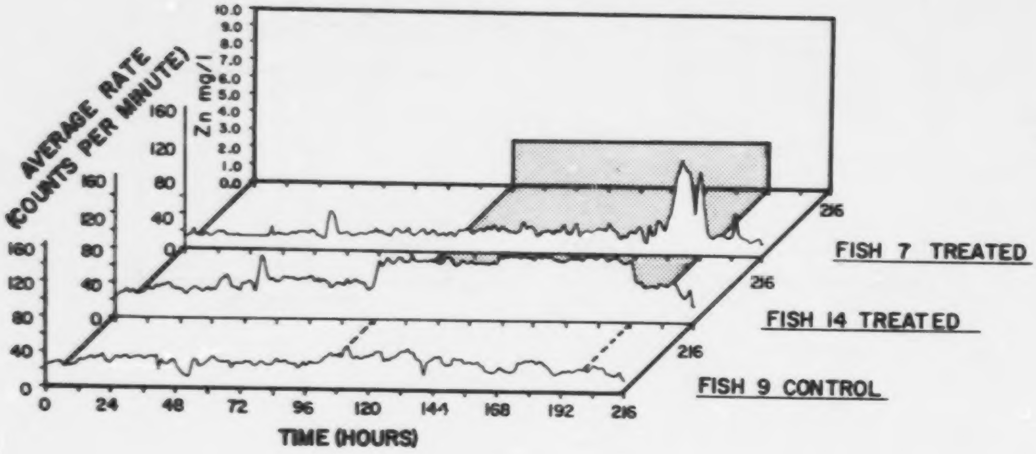
11. Monitoring system. Video signal from television camera enters both video-microprocessor interface and video monitor. Interface generates digital X and Y coordinates of points along length of object, and microcomputer controls sampling of coordinates, data manipulations, and storage using teleprinter and tape punch. Raw data are a time series of mean X and Y coordinates (time in seconds followed by a 3-digital octal Y coordinate and a 3-digit octal X coordinate).



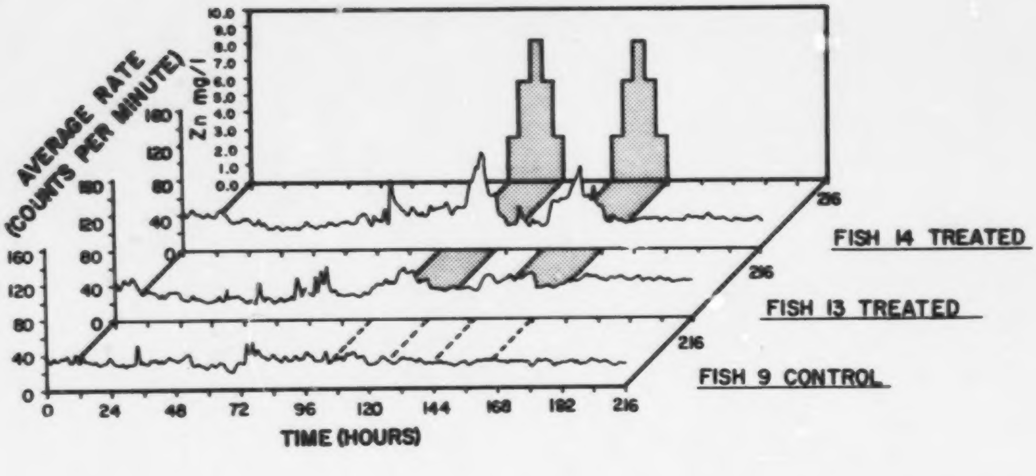
12. Changes of position of fish in the tank following exposure to a sublethal dose of toxicant.


DECHLORINATED TAP WATER 22°C
DILUENT WATER RESERVOIR
TOXICANT DELIVERY PUMP
TOXICANT RESERVOIRS
MIXING CHAMBERS
WATER DELIVERY CHAMBERS
ISOLATION ENCLOSURE AND FISH CHAMBERS
SAND FILLED BOXES
AMPLIFIERS
STRIP CHART RECORDER
TAPE DRIVE
OSCILLOSCOPE
MINICOMPUTER
COMPUTER TERMINAL
TO IBM/370-158

13. Schematic of variable dosing apparatus.



14. Ventilatory response of fish to constant concentration.



15. Ventilatory response of fish to pulsed concentration.

ALGAL CHLOROPHYLL MEASUREMENTS: TRADITIONAL AND UNTRADITIONAL APPLICATIONS OF A BIOLOGICAL SENSOR

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and

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INTRODUCTION

Characteristics of a useful biological sensor for water quality monitoring must include (1) biological and ecological significance; (2) sensitivity to environmental events; (3) simplicity of measurement; and, preferably, (4) suitability for automated in-situ determination. Measurements of photosynthetic pigments, particularly chlorophyll a, have long been recognized as valuable biological parameters for oceanographic and limnological investigations and water quality monitoring. Attributes which make chlorophyll determinations useful for these purposes include (1) the chlorophyll a molecule (fig. 1) which is the essential photocatalyst for the photosynthetic production of organic matter, and therefore, its measurement provides an indication of both algal biomass and potential production; (2) chlorophyll-bearing primary producers which form the ecological base of most marine and freshwater systems, and respond sensitively to environmental conditions by changes in cellular physiological status, productivity and/or biomass; and (3) algal photosynthetic pigments which can be extracted from mixed phytoplankton assemblages into organic solvents, and chlorophyll concentrations determined by spectrophotometric or fluorometric methods.

However, the amount of chlorophyll present per unit algal biomass is not constant, but varies with light intensity, cellular physiological status and the taxonomic composition of the algal assemblage sampled. Thus, chlorophyll determinations for ecological or water quality purposes should be considered only semiquantitative estimates at best. That such a semiquantitative indicator has received continued scientific application is testimony to the biological and ecological pertinence of chlorophyll measurements. If scientific progress was contingent on the availability of perfect methodologies, there would be very little of it.

Measurement of phytoplankton pigment levels, introduced by Harvey in 1934 (Vollenweider 1974), continues to be widely employed as an indicator of algal standing crop and potential primary production. Although considerable research effort has been invested in methodological modification, in-vitro pigment analyses (i.e., pigments extracted from the cells) have remained relatively unchanged during the past two to three decades (e.g., see the Report of the Committee on Chlorophyll from the international symposium, "Measurements of

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Primary Production in the Sea," Bergen, Norway, September 1957: Rapp. Proc.-Verb., Cons. Internat. Explor. Mer 144, 1958). However, significant progress has been made in applying fluorometry to both in-vitro and in-vivo chlorophyll determinations. Fluorometric techniques extend the sensitivity of in-vitro measurements, while determinations of in-vitro chlorophyll fluorescence (i.e., fluorescence from intact living cells) provide not only an index of phytoplankton biomass, but can also serve as an indicator of the physiological status of naturally occurring phytoplankton assemblages (e.g., Samuelsson et al. 1978; Cullen and Renger 1979; Kimmel and White, in press).

IN-VITRO CHLOROPHYLL METHODS

Spectrophotometric Measurements--The spectrophotometric determination of extracted pigment levels continues to be widely applied as a measure of algal standing crop. A variety of organic solvents (e.g., acetone, methanol, anhydrous ether, and dimethyl sulfoxide) has been employed in efforts to obtain sharper absorption peaks for the chlorophylls and accessory pigments (carotenoids, xanthophylls, and phycobilins). Individual investigators and international working groups have recommended slightly differing empirically derived formulae for estimating chlorophyll *a*, *b* and *c* concentrations based on the determination of absorbance at several wavelengths, usually 630, 645, 665 and 750 nm (e.g., Richards and Thompson 1952; Parsons and Strickland 1963, UNESCO 1966; and Jeffrey and Humphrey 1975). These procedures were developed for the purpose of distinguishing the contributions of chlorophylls *a*, *b*, *c*, and total carotenoids (which have overlapping absorption spectra) in a mixed pigment extract. Additionally, much effort has been spent evaluating various combinations of concentration and storage procedures, determining pigment extraction efficiencies as a function of solvent and extraction method, and establishing corrections for the occurrence of phaeopigments (e.g., Humphrey 1963, Marker 1972, Jeffrey and Humphrey 1975, and Shoax and Luim 1976).

Chlorophyll degradation products (phaeopigments) interfere with the spectrophotometric determination of chlorophyll concentrations by absorbing light in the same spectral region (663-665 nm) as chlorophyll *a*. However, interference due to the presence of phaeopigments can be corrected for by determining the relative changes in absorbance (or fluorescence) induced by acidification of the sample (e.g., Yentsch and Menzel 1963; Holm-Hansen et al. 1965; Lorenzen 1967; and Moss 1967). Such a variety of protocols for chlorophyll measurement has been proposed that standardization of procedures to permit comparison of results has become of more immediate need than development of the "ideal" technique. Most investigators in the U.S. now commonly employ the spectrophotometric and fluorometric methods described by Strickland and Parsons (1972) and Vollenweider (1974) for in-vitro determinations of chlorophyll concentrations.

Fluorometric Measuremenmts--Photosynthetic pigment extracts are fluorescent; when excited by short wavelength radiation in the blue region (430-450 nm), long wavelength radiation in the red region (650-680 nm) is emitted. An appropriate combination of excitation lamp, primary and secondary filters, and a red-sensitive photomultiplier permits fluorometric determination of extracted chlorophyll *a* with 100x greater sensitivity than by spectrophotometric methods (Kiefer 1973*a*). Kalle (1951, cited in Krey 1958), Yentsch and Menzel (1963) and Holm-Hansen et al. (1965) were the first to take advantage of the sensitivity and convenience of fluorometric methods for chlorophyll determinations in the field. Fluorometric techniques for chlorophyll and phaeopigment estimations are now used widely, particularly in studies of oligotrophic systems where the increased sensitivity provides maximum benefit. However, fluorometric measurements are in relative units and must be standardized against spectrophotometric determinations of "known" chlorophyll concentrations to provide quantitative chlorophyll estimates.

IN-VIVO CHLOROPHYLL METHODS

In-vivo Chlorophyll Fluorescence--The in-vitro chlorophyll techniques discussed above necessarily involve (1) collection and filtration of discrete samples, (2) grinding and extraction of samples in an organic solvent, and (3) determination of the absorbance or fluorescence of each extracted sample. Although significantly less time consuming and laborious than estimation of algal biomass by cell enumeration procedures, in-vitro methods are not readily applied to continuous or in-situ water-quality monitoring. Measurement of in-vivo chlorophyll fluorescence avoids the concentration and extraction requirements of in-vitro methods and thus makes continuous monitoring of algal chlorophyll levels feasible.

The characteristics of in-vivo chlorophyll fluorescence (maximum excitation at 436 nm, maximum emission at 685 nm) are only slightly different from those of chlorophyll in vitro. In-vivo chlorophyll fluorescence yield (fluorescence per unit extractable chlorophyll) is only about 10% that of chlorophyll dissolved in acetone; however, the availability of highly sensitive photomultipliers in modern fluorometers minimizes sensitivity loss. Lorenzen (1966) first proposed a method for the continuous measurement of chlorophyll levels and demonstrated the correspondence of in-vivo chlorophyll fluorescence and extractable chlorophyll for marine phytoplankton samples (fig. 2). Lorenzen's methodology (involving continuous pumping of unfiltered seawater through a standard fluorometer fitted with a large volume flow-through cuvette) has been widely applied in both marine and freshwater systems to the investigation of (1) horizontal chlorophyll distribution (e.g., Armstrong et al. 1967, Flemer 1969, and Caperon et al. 1971) and phytoplankton patchiness (e.g., Platt et al. 1970 and Powell et al. 1975) and (2) vertical chlorophyll profiling (e.g., Berman and Rodhe 1971, Kiefer et al. 1972, and Berman 1972).

Although continuous measurement of in-vivo chlorophyll fluorescence appears to provide an easily obtained real-time estimator of in-situ phytoplankton standing crop, the method is not without problems. The correspondence of in-vivo fluorescence to chlorophyll concentration is not constant but varies with (1) the recent light history of the cells, (2) algal physiological state as influenced by growth phase and available nutrients, (3) diel rhythms in photosynthetic activity, and (4) the taxonomic composition of the phytoplankton community sampled. Fluorescence yield (fluorescence / mg Chl a m^{-3}) has been observed to vary 2-5x under laboratory conditions (Kiefer 1973b, Slovacek and Bannister 1973, and Heaney 1978) and up to 10x in natural phytoplankton assemblages (Kiefer 1973c, Loftus and Seliger 1975, and Heaney 1978). Such variability makes necessary frequent comparisons of in-vivo fluorescence readings with extracted chlorophyll measurements to permit accurate data interpretation.

DCMU-Enhanced In-Vivo Chlorophyll Fluorescence--Photosynthesis researchers have employed the herbicide DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) in experimental investigations of the photochemical processes occurring in green plant cells for almost two decades. The maximal in-vivo fluorescence and lack of photosynthetic oxygen evolution observed when algae or other plant cells are exposed to light in the presence of DCMU has been attributed to DCMU-blocking of photosynthetic electron transport and the subsequent dissipation of much of the energy of excited chlorophyll molecules by fluorescence (Duysens and Sweers 1963).[1] Although the quantum yield of energy absorbed by the reaction centers of Photosystem II can be dissipated by several competing de-excitation processes (photochemistry, fluorescence from Photosystem II, spillover to Photosystem I, or radiationless transfer) a complementary relationship of photosynthetic activity and in-vivo chlorophyll fluorescence has often been observed (e.g., Butler 1966, Goedheer 1972, Papageorgiou 1975). Such correspondence suggests (1) a partial explanation for the variability of fluorescence yield observed for algal cultures and natural phytoplankton samples (i.e.; fluctuation in photosynthetic activity, thus variable in-vivo fluorescence per unit chlorophyll), (2) the use of DCMU to reduce the variability of in-vivo chlorophyll measurements, and (3) application of the variable fluorescence response (i.e., the relative fluorescence increase induced by DCMU poisoning) as an indicator of algal physiological status.

Slovacek and Hannan (1977) examined the applicability of DCMU-poisoning for maximizing the fluorescence yield of phytoplankton samples, and thereby reducing the variability of in-vivo chlorophyll fluorescence estimates of algal biomass. DCMU-poisoning of samples effectively reduced dependence of in-vivo chlorophyll fluorescence on environmental conditions and algal growth state, and decreased the variability of fluorescence yield (fig. 3). They concluded that DCMU-blocking of electron transport from Photosystem II[2] separates chlorophyll a from its physiological function, as does chlorophyll extraction into an organic solvent. However, other investigators have had more mixed results in applying DCMU-enhanced chlorophyll fluorescence to in-vivo chlorophyll measurements (e.g., Esaias 1978, McMurray 1978, Prezelin 1978, Frey 1979, and Slovacek 1978, 1979). Esaias (1978) observed little improvement in variability of fluorescence yield in a comparison of unpoisoned and DCMU-poisoned fluorescence measurements using parallel continuous-flow fluorometers. Slovacek (1978) noted similar difficulties, but attributed at least part of the variability to the continuous-flow system. White (1980) found that although DCMU-enhanced fluorescence provided increased analytical sensitivity at lower chlorophyll concentrations, the in-vivo fluorescence of both poisoned and unpoisoned samples was highly correlated with extracted chlorophyll a and, thus, either measurement was equally suitable as a biomass indicator.

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[1] DCMU prevents reoxidation of the Q intermediate and thereby blocks noncyclic electron transport from Photosystem II and the oxidation of water (i.e., the Hill reaction).

[2] At ambient temperatures, in vivo fluorescence emanates primarily from chlorophyll a associated with Photosystem II; Photosystem I is only weakly fluorescent.

Samuelsson and Oquist (1977) and Heaney (1978) suggested that the variable nature of in-vivo chlorophyll fluorescence might be used to an advantage in the investigation of algal physiological state in situ. Halldal and Halldal (1973) had specifically indicated the potential usefulness of DCMU-enhanced in-vivo chlorophyll fluorescence for phytoplankton studies. By virtue of the approximate complementarity of photosynthetic activity and in-vivo chlorophyll fluorescence, an indication of the physiological status of a phytoplankton assemblage should be possible by comparison of the in-vivo fluorescence (unpoisoned) and the maximum (DCMU-enhanced) fluorescence. Samuelsson and Oquist (1977) and Samuelsson et al. (1978) reported the correspondence of DCMU-induced fluorescence increases and photosynthetic ^{14}C uptake in algal cultures, and further emphasized the potential applicability of the method to the investigation of natural phytoplankton communities.

A direct technique for assessing the physiological status of naturally occurring phytoplankton assemblages in-situ would indeed be valuable for examining factors influencing phytoplankton productivity, biomass accumulation, spatial distribution, and bloom development and decline. Currently, it is necessary to make both primary productivity and extracted chlorophyll measurements to obtain data on phytoplankton physiological state or photosynthetic potential. Therefore, we conducted a series of laboratory and field evaluations of the DCMU-enhanced in-vivo fluorescence response as an index of phytoplankton physiological status. Although some of our results are being reported elsewhere (Kimmel and White, in prep.), they are not yet available in published form and therefore will be summarized here.

We first tested the correspondence of fluorescence response and photosynthetic ^{14}C fixation in laboratory light-deprivation experiments designed to simulate the gradual physiological decline experienced by phytoplankton cells introduced to aphotic depths (fig. 4). The fluorescence response index (FRI) proved to be directly related to photosynthetic carbon fixation in these experiments (fig. 5), thus indicating that the fluorescence response reflects changes in phytoplankton photosynthetic potential, at least in the laboratory.[3] Subsequently, vertical profiles of in-vivo chlorophyll fluorescence (F_i), DCMU-enhanced fluorescence (F_d), and FRI in several Oklahoma reservoirs revealed differences in phytoplankton physiological status, apparently related to differing conditions of light and nutrient availability and vertical mixing patterns (figs. 6 and 7). In-situ sample displacement experiments conducted in conjunction with primary productivity measurements provided field evidence that FRI values accurately reflected phytoplankton photosynthetic potential (table 1).[4] Fluorescence response measurements recently conducted on near-surface samples taken along a well-established nutrient availability gradient in Lake Mead

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[3] Fluorescence response index = FRI = $(F_d - F_i) / F_d$; where F_i = initial (unpoisoned) in-vivo fluorescence and F_d = DCMU-enhanced in-vivo fluorescence. FRI has a theoretical range of 0.0 (i.e., no photochemical electron transport) to 1.0 (i.e., all absorbed energy dissipated photochemically), but usually ranges from 0.2 to 0.7.

[4] Samples obtained from several depths were incubated at a single depth to compare their photosynthetic potentials under similar light and temperature conditions.

Table 1.--Comparison of phytoplankton photosynthetic potential as reflected by photosynthetic carbon uptake (C uptake / F_d) and DCMU-enhanced in-vivo chlorophyll fluorescence (as FRI) in Lake Texoma and Broken Bow Lake sample displacement experiments. Incubation depths were 1 m (520 uE m^{-2} sec $^{-1}$) and 3 m (300 uE m^{-2} sec $^{-1}$) in Lake Texoma and Broken Bow Lake, respectively. F_d, which reflects algal chlorophyll, is expressed in relative units (counts sec^{-1}). From Kimmel and White (in press).

Sampling Depth (m)	Fd	C Uptake ($\mu g\ C\ l^{-1}\ hr^{-1}$)	FRI	$\frac{C\ Uptake}{F_d}$
Lake Texoma, 26 Jul 1978:				
1	226	46.86	0.51	0.21
3	215	43.25	0.50	0.20
8	202	44.66	0.50	0.22
Broken Bow Lake, 14 Aug 1978:				
3	19	4.04	0.38	0.21
8	47	25.14	0.41	0.54
12	30	18.89	0.46	0.64

(Nevada-Arizona) ranged from 0.39 to 0.63 (as FRI) and were directly related to available nutrients and carbon fixation rates (Kimmel, unpublished data). Additionally, it appears that fluorescence response measurements may be applicable to algal bioassays. Preliminary tests show FRI determinations to be a sensitive and convenient means of assessing phytoplankton responses to experimental nutrient and silt additions. The same should hold true for toxicity bioassays, although we have not yet explored this potential application.

Our results suggest that the chlorophyll fluorescence response technique provides a rapid and convenient method for obtaining information on the in-situ physiological status of naturally occurring phytoplankton assemblages, information otherwise obtainable only by measuring both primary productivity and extracted chlorophyll. However, in-vivo chlorophyll fluorescence is still a poorly understood phenomenon, and its application to field investigations and environmental monitoring is not free of uncertainty. Diel fluctuations in fluorescence response and photosynthetic activity (Prezelin and Sweeney 1977; and Prezelin and Ley, in press), the relationship of F_i and F_d to extractable chlorophyll levels (McMurray 1978, Esaias 1978, and White 1980) effects of algal community species composition (Kiefer 1973c, Heaney 1978, and White 1980) and high light intensities (Vincent 1979, and G. P. Harris, pers. commun.) on in-vivo flueorescence and fluorescence response measurements all require further clarification. At present, DCMU-enhanced in-vivo fluorescence data must be interpreted cautiously and applied only as a relative indicator of algal biomass and physiological status.

Our initial evaluation of the fluorescence response technique and its application in our limnological studies has produced a sense of cautious optimism in regard to future applications of the method. The simplicity and convenience of the fluorometric analysis, the ecologically integrative nature of the information obtained, and the potential of the technique for automated in-situ water quality monitoring comprise strong arguments for continued evaluation and development of in-vivo chlorophyll fluorescence response as a biological sensor.

SUMMARY AND PROSPECTUS

Chlorophyll a is essential for the photochemical conversion of radiant energy to the potential energy represented by organic compounds. As such, estimates of chlorophyll levels present in marine and freshwater systems have long been employed as a biological sensor of algal biomass and potential photosynthetic production. Spectrophotometric and fluorometric determinations of extracted chlorophyll a will continue to be widely used as indicators of phytoplankton standing crop; however, in-vivo fluorescence methods provide greater potential for in-situ monitoring purposes.

Determination of horizontal and vertical phytoplankton distributions and automated continuous monitoring of algal chlorophyll levels at stationary points are possible using continuous-flow fluorometry. Mapping of longitudinal chlorophyll gradients in near-surface water with continuous-flow systems provides a means of obtaining, on a frequent basis, the real-time ground-truth data required for evaluating the utility of satellite remote sensing for water quality monitoring. The combination of in-vivo chlorophyll fluorescence and DCMU-enhanced fluorescence determinations provides an indication of both algal biomass and physiological status. Thus, the fluorescence response technique represents (1) a significant increase in the information content of chlorophyll measurements and (2) a high potential for application to automated in-situ monitoring of algal communities. In-vivo chlorophyll fluorescence is a complex and poorly understood process; however, the sensitivity, convenience, and information content of fluorescence response measurements suggest that the applicability of the technique for water quality monitoring purposes should be thoroughly explored.

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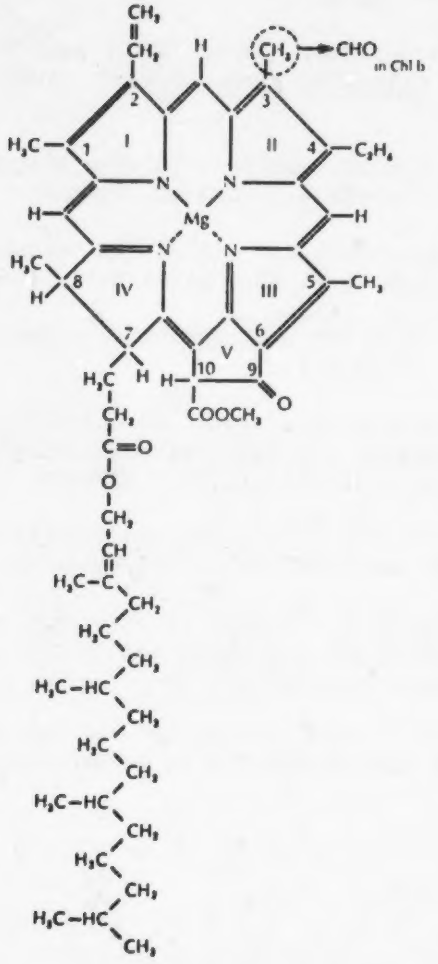
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Figure 1. The chlorophyll *a* molecule

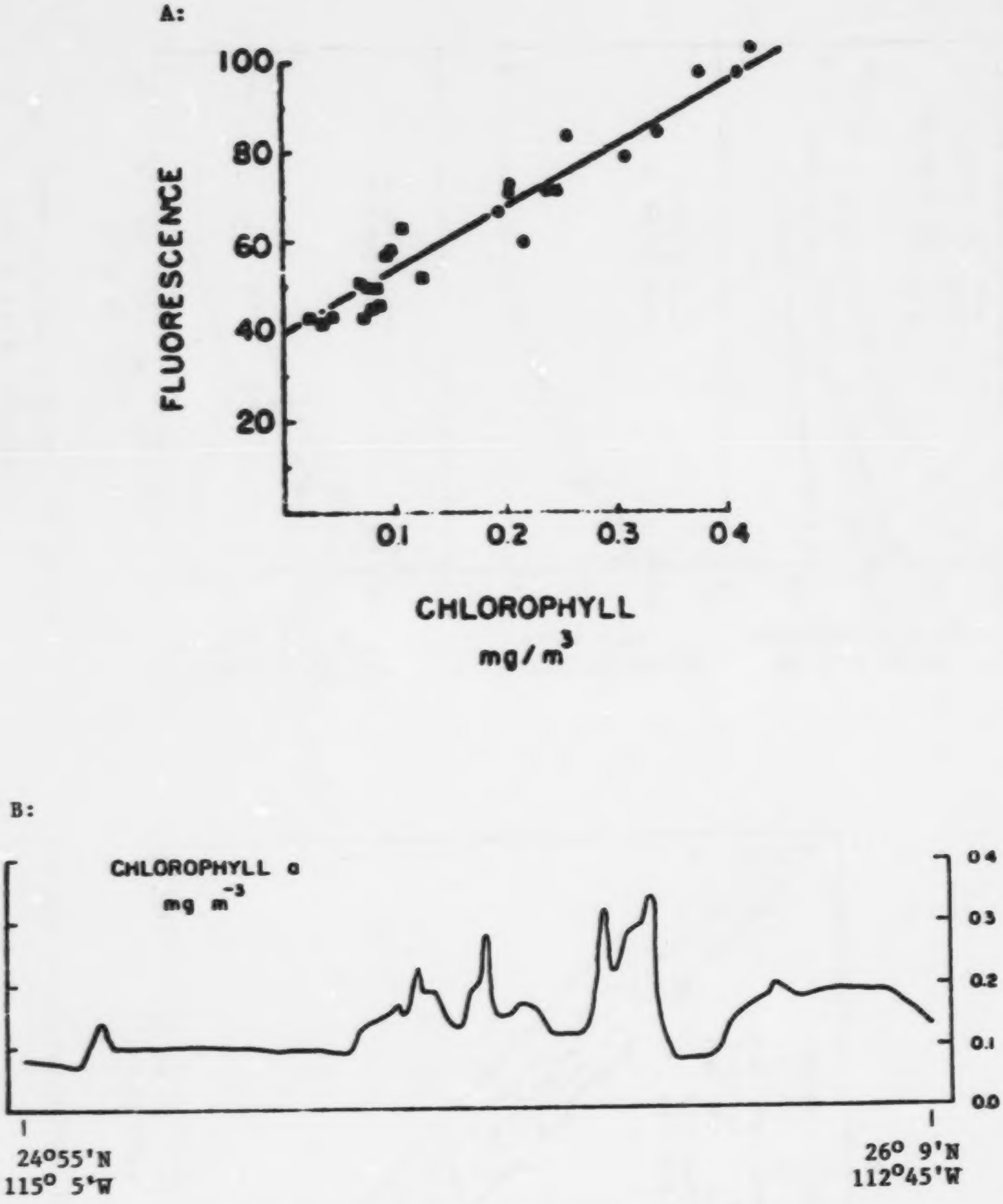

Figure 2. Correspondence of in-vivo chlorophyll flourescence and extractable chlorophyll

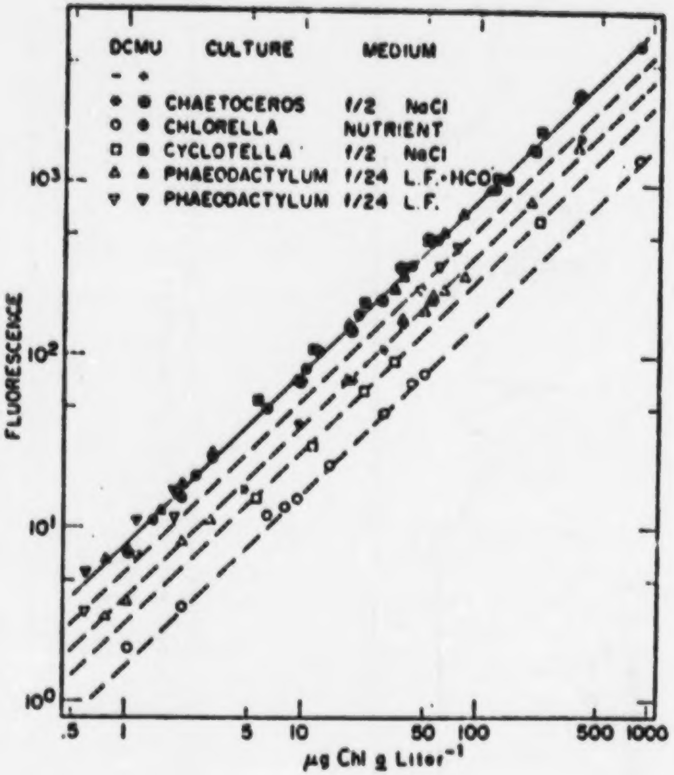

Figure 3. Effect of DCMU-poisoning of samples

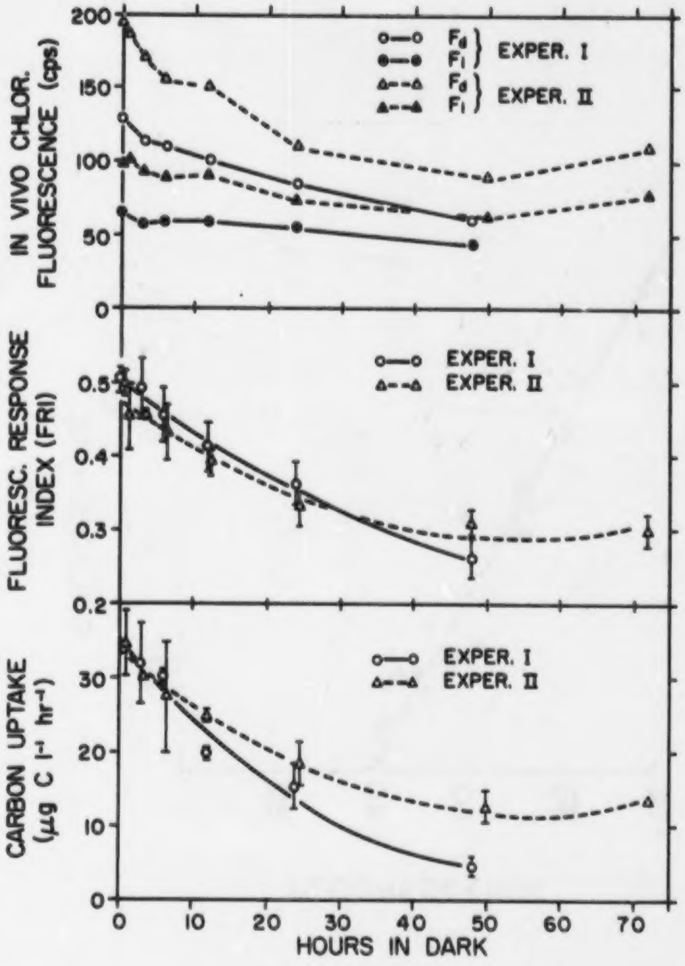

Figure 4. Effect of aphotic depths on phytoplankton cells

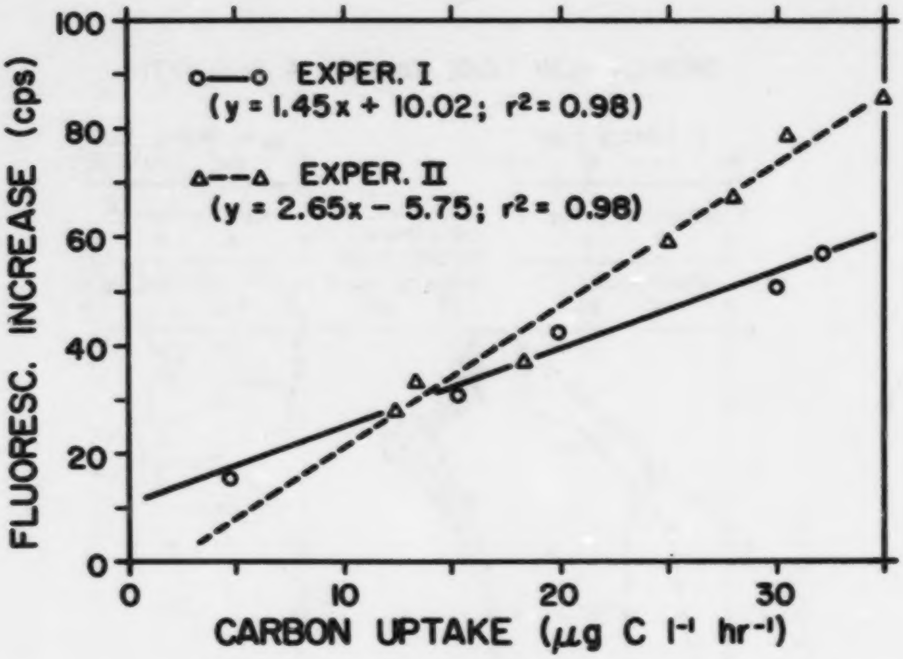

Figure 5. Photosynthetic carbon fixation effect on flourescence response index

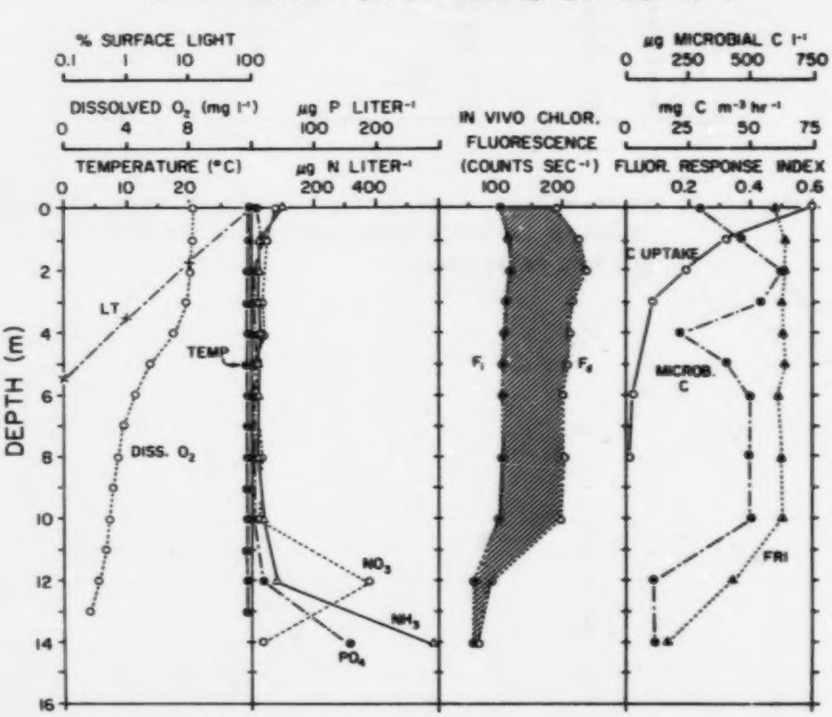
LAKE TEXOMA (OKLA.-TEXAS): 26 JULY 1978

Figure 6. Difference in phytoplankton physiological status related to differing conditions of light and nutrient availability

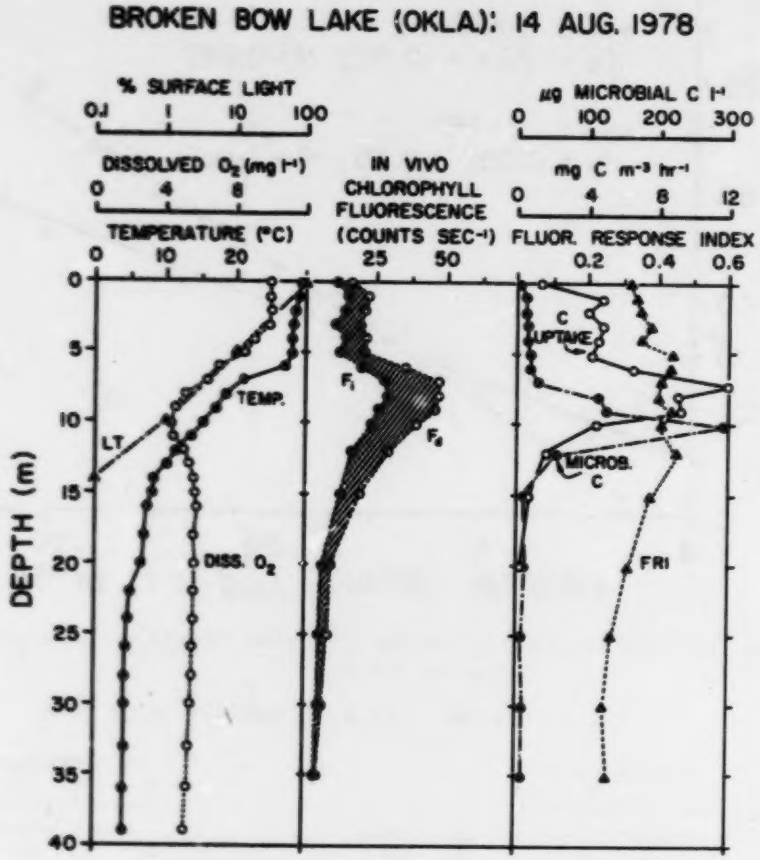

Figure 7. Difference in phytoplankton physiological status related to difference condtions of vertical mixing patterns.

METABOLIC RESPONSE OF FISH TO STRESS[1]

by

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INTRODUCTION

The fact that humans are primarily terrestrially oriented leaves the aquatic environments, especially marine water, out of sight and out of mind altogether too often in terms of recognizing effects of, and coping with, waste disposal. The lack of public awareness of effects of wastes on marine organisms is commonplace. Indirect observations of stresses on marine biota are often recognized only after major damage has occurred -- such as when a fishery fails. When a fishery failure, for example, has been recognized, it is difficult to decide whether the causative factors involved overfishing, pollution, naturally fluctuating fish populations, or naturally fluctuating environmental variables. Early warning and monitoring systems are needed.

At the outset of a marine pollution study there is usually little definition of the extent of toxicity, although a facile assumption may be made that mobile organisms like fish would simply swim to less toxic areas. Unfortunately there is little direct evidence for this behavior except in laboratory studies; more likely, there is little evidence to indicate whether fishes have the sensory mechanisms to perceive anthropogenic materials in the first place and whether they would react solely, and negatively, to such materials in the second place. Many other favorable environmental factors like availability of food, spawning grounds, etc., might well mask unfavorable factors such as low levels of toxic materials. These types of organismal, population, or ecosystem response problems exist over and above the usual problems of pollution detection.

Synthesizing existing information and providing techniques for the solution of specific problems of toxicity in open natural systems have been and remain elusive (NAS 1975). Historically, pollutants have first been identified after some environmental--usually biological--adversity had become obvious, and then the nature of the pollutant and its biotic effects would have been determined. The delay in assessing effects of pollutants on biological systems and the primary emphasis on identification of the pollutant has been the subject of some concern (NAS 1975, Brungs and Mount 1978, among others).

To cope with these problems many authors and some agencies have suggested various schemes for biomonitoring, e.g. Cairns, _et al_. (1973a,b) or Cairns, _et al_. (1979). The magnitude of problems of estimating hazards has been emphasized by Cairns (1980). With this type of emphasis, we can turn to a simple method of measuring what fishermen might perceive early on when fish are subjected to stresses.

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[1] The University of Texas Marine Science Institute Contribution No. 000

Quite often fishermen recognize subtle adverse environmental effects on fishes long before acute effects are obvious and are generally recognized by various governmental agencies concerned with environmental quality. Why is this possible? Why should not whatever aspects of the fish or the environment that the fisherman can perceive as stress be utilized in an analytical manner?

The answers to these questions turn out to be rather straightforward. And the answers to how stress effects can be recognized can provide a rather elegant, quantitative method of bioassay by utilizing fishes as sensors of sublethal stress. Essentially the method is one of evaluating the decline with stress in metabolic output for sustained swimming performance, which was proposed by Fry (1947) and originally utilized by him and others chiefly to determine effects of thermal stresses (Fry, 1957, 1971).

Emphasis throughout will be on sublethal stress effects. Acute stresses that produce mortality rates as high as those of 96 hr LC_{50} evaluations will not be discussed, for the reason that even at 1% of such high mortality rates, the natural mortality rates of many longer lived fish species would be excessive for population survival. The sublethal stress effects should be recognizable and measurable with precision at about 1% of the LC_{50} level or less. Techniques should be adaptable to open environmental systems and to endemic fishes as well as being adaptable to laboratory or treatment plant situations. Most importantly emphasis should be on a technique that does not necessarily depend on a priori knowledge of the chemical nature of toxicants.

The measurement of subtle, sublethal stress effects should be sufficiently easy, yet sensitive enough so that true predictivity is possible long before any acute damage would be evident. Measurements should be sensitive for assay purposes; they should be easy in the sense that complicated procedures requiring extensively trained personnel and costly equipment are avoided; they should be easy also in the sense that interpretation of results are straightforward and conceptually in line with pertinent real-world biotic and environmental features.

PURPOSE

The purpose of this presentation is to show by selected examples and general theoretical considerations how fish can be used to

1. Monitor consistency of water quality;

2. Establish optimal natural environmental conditions;

3. Assess and monitor normal, or sublethal departures from normal, conditions with respect to optimal natural environments;

4. Assess and monitor deleterious sublethal stress effects of toxic materials; and

5. Predict long term outcome of natural or anthropogenic stresses at sublethal levels.

With these aims in mind, the term "natural environment" may mean "ambient environment" if man-induced environmental modifications persist.

WHY UTILIZE FISHES?

The use of fishes for biological assays and for monitoring involves ecological considerations of both theoretical and practical importance. Examples of these considerations are noted below.

1. Fishes are widely known to the public at large as commercial and recreational resources.
2. Fishes are usually much better known taxonomically than other groups of organisms.
3. Among fishermen and biologists there is a considerable amount of "native wisdom" on the nature of fishes and their sensitivity to environmental variables.
4. There is a large literature on the highly sensitive reactions of fishes to natural and anthropogenic environmental factors, although few baselines have been established.
5. Fishes are useful comparatively in assessing environmental conditions because, more than almost any other taxonomic group, they are found in nearly all marine environments.
6. Throughout the world fishes tend to have rather similar physiological systems that can be compared among themselves with reference to adaptational propensities to specific environments; the ubiquitous distribution of marine fishes implies that they can be compared from one type of environmental regime to the next in terms of physiological characteristics.
7. Fishes in a given environment tend to have an ecological stability that assures survival over relatively long periods and at relatively constant numerical and biomass levels, compared to most other aquatic organisms. These levels can naturally vary usually less than, say, one order of magnitude over periods of decades, whereas short-lived species, like microorganisms at lower ecotrophic levels can naturally vary, say, ten orders of magnitude in a few hours or days in response to natural environmental changes.
8. Because most fish species are at the higher ecotrophic levels and tend to have relatively stable populations, their stabilizing and integrating effects on the overall natural ecosystems are most likely highly important.

It is generally known--at least by hindsight--that all the above considerations are affected by both acute and chronic stresses on fish populations, whether these stresses are natural or anthropogenic.

BASIC THEORY

Fry (1947) noted that the respiratory metablic scope for activity is a sensitive measure of well being in the case of natural variables like temperature. Metabolic scope is simply the difference between the aerobic metabolic rate at sustained swimming speeds and the rate at a maintenance, or standard, level (fig. 1). Respiratory metabolism is most commonly measured by determining the rate of consumption of dissolved oxygen by fish in a variety of ingeniously constructed chambers. Fry (1971) illustrates some of the more common types of respiration chambers and discusses many of the techniques for the determination of respiratory rates of both standard and active levels.

Determination of respiration rates at standard conditions involves many known pitfalls, most of which are avoidable with relatively simple precautions. These precautions are usually to assure that the rates are measured on fasted fish and are the lowest possible rates compatible with survival for several days. Fry (1957, 1971) and other authors have covered this subject extensively. The standard rate can not decrease, but it can increase if energy costs for internal regulation are required for ordinary natural stresses like temperature and salinity. The standard level may or may not increase with anthropogenic stresses, presumably depending whether or not the body "machinery" has any available adaptational mechanisms for the particular stress.

The metabolic rate at maximum sustained swimming activity of a fish usually involves the oxygen consumption rate measurements for a speed of swimming that can be maintained without fatigue for long periods, say, 200 minutes or longer according to Beamish (1978) in a review of the entire subject of swimming capacity. Observations on Gulf coastal fishes reveal that with conditioning at about 1 length/sec for about 12 hours and gradually increasing the swimming speed of a fish to its maximum in about 10 cm/sec increments each 5 min would produce highly reproducible results. Further if fish swam at even a few cm/sec over a maximum sustained rate, they would either tend to tire (and probably build up an "oxygen debt" with attendant anaerobic respiration) or they would initiate "burst-and-glide" swimming. However, after performing at the maximum rate for several hours, the same aerobic performance could be repeated, but not exceeded, with a short rest and no evidence of fatigue. The repeatability and the fact that the maximum sustained swimming performance has a sound basis in both physiological and hydrodynamic theory (Webb 1975, Webb 1978, and Magnuson 1978) make this measurement exceptionally useful.

From figure 1, general theory of energetics and physiological performance, and practical experience, the logical possibilities of sublethal stress effects reduce the scope for activity and involve raising the standard level, or lowering the sustained active level, or both. Recently, Wohlschlag and Parker (ms) noted that diluted pharmaceutical industry wastes could slightly elevate active metabolic levels; although the swimming rates declined substantially, the standard metabolism increased and the fish showed incipient morbidity after about 2 days. This kind of elevated metabolism brought about by stimulatory effects of drugs, but not accompanied by locomotor activity, is possibly a kind of "spontaneous activity" (Fry 1957).

Spontaneous activity must be eliminated for the measurement of standard rates; but it ordinarily is trivial if fish will swim at maximum sustained rates. Several other higher levels of swimming activity and corresponding metabolic rates that should be mentioned are the shorter duration (20 sec - 200 min) higher prolonged rates and the very short duration (less than 20 sec) very high burst rates. Usually averaging less than the maximum sustained active rates are the routine rates that apply to foraging, schooling, migrating, station holding, and other swimming requirements. These other rates are defined and discussed in detail by Beamish (1978), of which only routine rates are important to the subject at hand inasmuch as they also tend to be depressed by stresses (Beamish 1964 and Wohlschlag and Cameron 1967).

TECHNIQUES

There is a large literature describing a variety of devices used for measuring the respiratory metabolic (oxygen consumption) rates of fishes at various stages of quiescence to extreme burst activities. A recent volume on the subject (Hoar and Randall, eds. 1978) has a number of chapters on technical and conceptual aspects of locomotion. Webb (1975) and Aleyev (1977) consider many hydromechanical and technical features. For this discussion, techniques used recently with Gulf coast fishes for the determination of metabolic scope will be emphasized (Wohlschlag and Wakeman 1978).

Acclimation--Fish used for metabolic measurements should be acclimated at a constant temperature and salinity for at least two days with fasting at a range of 15-30°C and about 10-50 ppt salinity with no 2-day change much over a 5° C or 10 ppt salinity change from ambient to experimental conditions. At colder temperatures, longer periods are desirable. Fish used for activity measurements and for sublethal toxic material responses should be held for another two days under about 1 L/sec swimming acclimation or with the toxic material. (For toxic materials a 1% dilution of an LC50 level, if known, is a good rule-of-thumb initial concentration1)

Standard Metabolic Rates--Standard oxygen consumption rates can be determined directly by holding the fish in partially darkened and sealed containers that are protected from visual and other disturbances, starting with measured oxygen concentrations and continuing until the level would be no lower than about 70% saturation.

Another method is to utilize appropriate sizes of tubes that are closed at each end, except for inlet and outlet tubes through which flows water whose oxygen contents and flow rates are constantly monitored. In this and the static system, the lowest rates consistently observed on a diel cycle correspond to the standard rates, but only if the fish are absolutely quiescent and show no evidence of spontaneous (nonlocomotory) activity.

A third method is to plot oxygen consumption rates (usually per kg of fish weight) at various activities and calculate the resting rate at zero velocity. (See methodology below.) If the regression data show the usual scatter, a line drawn parallel to this regression through the lowermost points and extrapolated to zero velocity will usually be a better estimate of the standard rate. This method is known as the Brett (1964) method.

Routine Metabolic Rates--These rates can be measured in an annular chamber that is rotated at whatever velocity the fish will swim consistently for a period of an hour or so. Oxygen content of the chamber should be measured initially and about each 15 min thereafter, but the minimum level should be no lower than about 70% for most active species. This method follows that of Wohlschlag and Juliano (1959), Wohlschlag and Cameron (1967), Wolschlag, et al. (1968) and Wohlschlag and Cech (1970) in which the chamber is immersed in the large tank where the fish have been acclimated. The technique obviates the disturbing effects of transferring fish from tank to metabolism chamber. Swimming speeds are determined by counting the revolutions and multiplying by the approximate swimming track circumference of the chamber over a given time period. Usually, the actively swimming species will swim at a routine rate about equal to 1 L/sec, which tends to be equivalent to a common speed for schooling, foraging, etc., although by rotating the chamber at higher and higher speeds, some species may be induced to maintain much higher velocities. Comparisons of routine rates with standard and active rates are in Wohlschlag and Wakeman (1978). Routine scope can also be utilized (Beamish 1964).

Active Metabolic Rates--Metabolic rates at maximum sustained swimming velocities (active rates) can be measured by a variety of respiratory chambers. Some of the more successful types are the rotating annular or toroidal types, the Brett swimming tunnels (Brett 1964), and the Blazka swimming tunnels (Blazka, et al. 1960). Fry (1971) illustrates some of these types of respirometers. For the studies described here a large Blazka-type chamber was constructed in such a way that it could be suspended in the water used for acclimation of the fish. Details are in Wohlschlag and Wakeman (1978) and in Wakeman and Wohlschlag (1978), from which the schematic figure 2 shows the essential working relationships. The suspension of the chamber on a trolley and the quick-disconnect feature for addition and removal of fish is a major feature. Direct measurement of swimming speeds is by utilizing a paddle type of electronic transducer speedometer inserted into the respiratory chamber access tubes. Indirect measurement of swimming speeds is by tachometer readings of the impeller shaft rotational speed which have been calibrated to direct measurements. Swimming speeds depend also on calibrations for water density that varies with temperature and salinity.

Calculations--Because experience has indicated that the weight of the fishes contributes about 80% of the variability to oxygen consumption rate measurements when stepwise regression procedures are employed, it is usually not desirable simply to express metabolic rates as O_2 consumed per unit weight per hr as is customary in many laboratory studies. Also, because it may be desirable to measure metabolism over a range of body weights, swimming velocities, temperatures, salinities or other variables, the use of multiple regressions is indicated in the form

$$\hat{Y} = a + b_w X_w + b_v X_v + \ldots + b_i X_i + \ldots ,$$

where

$\hat{Y}$ = expected rate of O_2 consumption in $\log_{10}$ mg O_2 /hr;

a = constant

X_w, X_v and other terms are independent variables, respectively for $\log_{10}$ weight in grams, for swimming rate in L (cm)/sec or in $L^{1/2}$/sec whichever may be linear, etc. for other variables; and b_w and b_v are the respective partial regression coefficients.

This procedure is described in most statistical manuals (e.g. Snedecor and Cochran 1967) or pretested library computer routines; it is the type of procedure used by Wohlschlag and Wakeman (1978) in which salinity X_s was used at higher orders. The procedure enables comparison at equivalent weight, activities, etc. providing extrapolation is not carried beyond the range of the observations.

EXAMPLES OF STRESS EFFECTS ON METABOLIC SCOPE

Temperature--Scope tends to be maximal at the optimal temperature, which for the spotted sea trout in figure 3 is probably around 23°C, although for many species scope may increase almost to the upper lethal level. Excellent reviews of temperature effects on fish physiology are in Brett (1970, 1971).

Salinity--Scope for the spotted sea trout, a coastal and bay species, can be described over a salinity range by the data of Wohlschlag and Wakeman (1978). Quite obviously in figure 4 the greatest scope occurs around 20-25 ppt salinity which represents a salinity optimum for juvenile to adult fishes. Note the one point at 30, and perhaps 35, ppt salinity where the active metabolic rate is low; this rate is most likely caused by poor growth and partially starved fish. Note also that the routine metabolic rate (at a swimming speed of about 1 L/sec over the salinity range) is equal to the active rate at extremes of about 10 and 45 ppt salinity. This is the salinity range where small to large spotted seatrout can be caught in the south Texas area. Also the species has not yet been successfully cultured in freshwater situations as have some of the other sciaenids.

In figure 5, the optimum of the scope corresponds almost exactly with the maximum swimming rate. This observation suggests that direct observations of the maximum swimming rates could be utilized for monitoring purposes. Fishermen generally recognize the fact that healthy fish swim more vigorously than those under prolonged stress.

Growth--After discovering that the active metabolic rates and the scopes were unusually low (Fig. 4) at 30 ppt salinity, it became obvious that the spotted seatrout for this portion of the experiment had been unusually slender. The fact that this species in mid to late summer in this area was not commercially marketable and the fact that the weight-length regressions of the experimental fish also indicated that they were considerably more emaciated as they became larger indicated their poor condition as in the figure 6, which shows the metabolic characteristics of the fish with a very poor condition compared to the average healthy fish at well fed and rapidly growing conditions. Actually under chronic stress, one of the first manifestations to be noticed in a fishery is that the so-called condition of the fish declines. This problem has been discussed by Cech and Wohlschlag (1975).

Red Tide Exposure--In one case of a spotted seatrout study, waters from Nueces Bay were transported to the Port Aransas Laboratory for comparison of routine metabolic performance. After this study had started there was evidence that a small red tide (*Gonyaulax monilata*) bloom had occurred in the Corpus Christi ship channel and some considerable amounts of this water, used as cooling water, had been discharged into Nueces Bay near the water collection site. As indicated in figure 7, the standard level increased in this water, so that if the active level, which was not measured then, was assumed to be at the same level as that for control fish (studied a short time later), the scope would be reduced as indicated.

Unknown Chronic Pollution--Samples of water from four Galveston Bay stations were periodically transported to the Port Aransas Laboratory for the study of striped mullet routine metabolism during 1971-72 (Wohlschlag, et al. 1973). The results of these measurements compared to those made for fish in "clean" Port Aransas area water are presented in figure 8. In all cases, the routine levels were slightly depressed in the Galveston Bay waters, which in spite of proximity to the highly polluted Houston Ship Channel are among the more productive coastal waters of the northwestern Gulf of Mexico.

Ocean Dumped Petrochemical Waste--Figure 9 shows how a low dilution of 0.2% v/v (about 1% of LC_{50} levels) of a well-weathered and deactivated waste from the biosludge ponds at the Shell Plant in Deer Park, Texas affected the metabolic scope of the red snapper at 20° and 28°C. Both liquid and sludge portions were evaluated at 20°C (Wohlschlag and Parker 1980, in press). The Gulf dumping program ceased before the procedures could be refined to utilize longer exposure periods at much lower concentrations. It would be desirable to investigate this problem with potential pollutants at levels below those considered as legally toxic by present standards.

Ocean Dumped Pharmaceutical Waste--In figure 10 are some results of an experiment to ascertain the scope diminishing effects of partially decomposed composite waste of the type dumped north of Puerto Rico (Wohlschlag and Parker ms). The waste held and transported with considerable delay at high summer temperatures was slightly more noxious in glass contaners than in polyethylene containers.

An additional series of experiments was conducted with the Puerto Rican composite and the individual pharmaceutical wastes at fairly low concentrations and exposures 2 hours or less, followed by a recovery period in clean water for several days (fig. 11). During the recovery period, average active metabolic rates and scope values were determined. In all cases the standard level tended to increase, and the swimming rate tended to decrease considerably, but the active metabolic rate in case of some of the individual pharmaceuticals was apparently stimulated to the extent that most of the fish showed signs of morbidity after about two days of the recovery period. This type of experiment needs further investigtation at lower exposure concentrations and times. Otherwise the experiments with longer times and still lower concentrations than those illustrated in figure 10 should be conducted for any persistent pollutant, whether or not its chemical nature is understood at the outset.

SUGGESTED PILOT MONITORING PROGRAM

From the preceeding data it is clear that where there may be even very slight persistent stresses, the metabolic scope of fishes and their swimming performances decline in proportion to the stress and all its associated chronic effects. Further, it is important to note that this technique has a very firm physiological basis and requires no a priori knowledge of the chemical nature of toxic materials or the biochemistry of toxicological effects on organisms.

It would be possible in a monitoring operation to utilize more or less continuous operations to measure swimming rates and general swimming performances both in flumes that contain clean water and in flumes to which suspected toxicants could be added for uniform concentrations. For metabolic rate determinations, there should be no problem to divert waters from the flumes to enclosed Blazka-type chambers for active metabolic rate determinations or to flow-through/static tubular chambers for standard metabolic rate determinations. (See the Cairns references.) All that would be required is for the Blazka chamber end dome opposite the dome that contains the impeller to be equipped with an inflow port and valve. By surrounding the Blazka and static chambers with the experimental or control waters, fish could be continuously acclimated and ready for experimentation at any time.

It should be emphasized that such methods of holding the fish also permit the type of monitoring suggested by Cairns and his coworkers (Cairns, Sparks, and Waller 1973; Cairns, Dickson, and Lanza 1973; and Cairns and Gruber 1979) because much of the same apparatus can be used. A most useful feature of the equipment for continuous observations of swimming rates and the equipment for holding the fish during the standard metabolic rate measurements is that a large category of behavioral observations can also be made. Many behavioral observations undoubtedly can be directly associated in quantitative terms with the degree of stress and depression of metabolic scope and swimming performance.

Perhaps the most biologcally useful attribute of performance energetics is the pertinence of their measurements to the general bioenergetics of fish populations. Recent references would include discussion by Webb (1978), Brett (1979), and Brett and Groves (1979). From these references, it will be clear that metabolic scope and swimming performance data used for biomonitoring or stress assessment all tie in with the generalizing concepts that bioenergetics embrace.

ACKNOWLEDGMENTS

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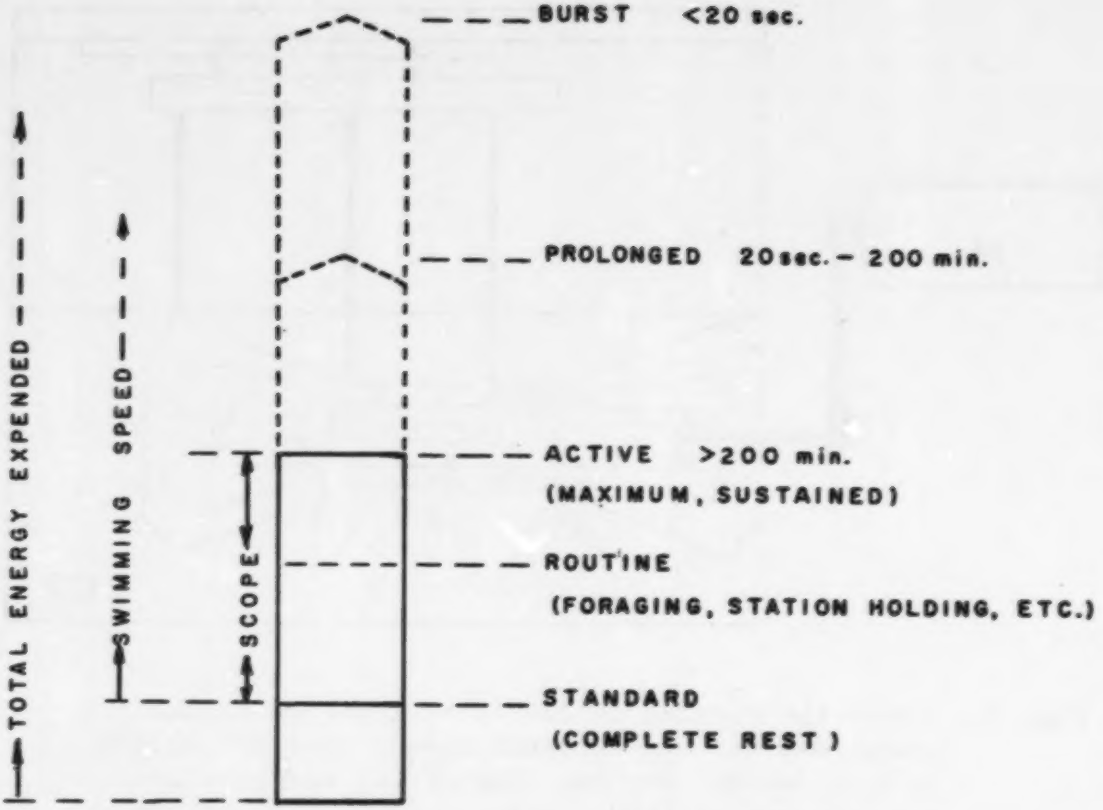

BURST <20 sec.
PROLONGED 20 sec. – 200 min.
ACTIVE >200 min.
(MAXIMUM, SUSTAINED)
ROUTINE
(FORAGING, STATION HOLDING, ETC.)
STANDARD
(COMPLETE REST)
TOTAL ENERGY EXPENDED
SWIMMING SPEED
SCOPE

Fig. 1. Relationships of energy levels and swimming speed levels. Note that scope can only diminish with stress because either the standard (minimum maintenance) level increases, or the active level decreases, or both.

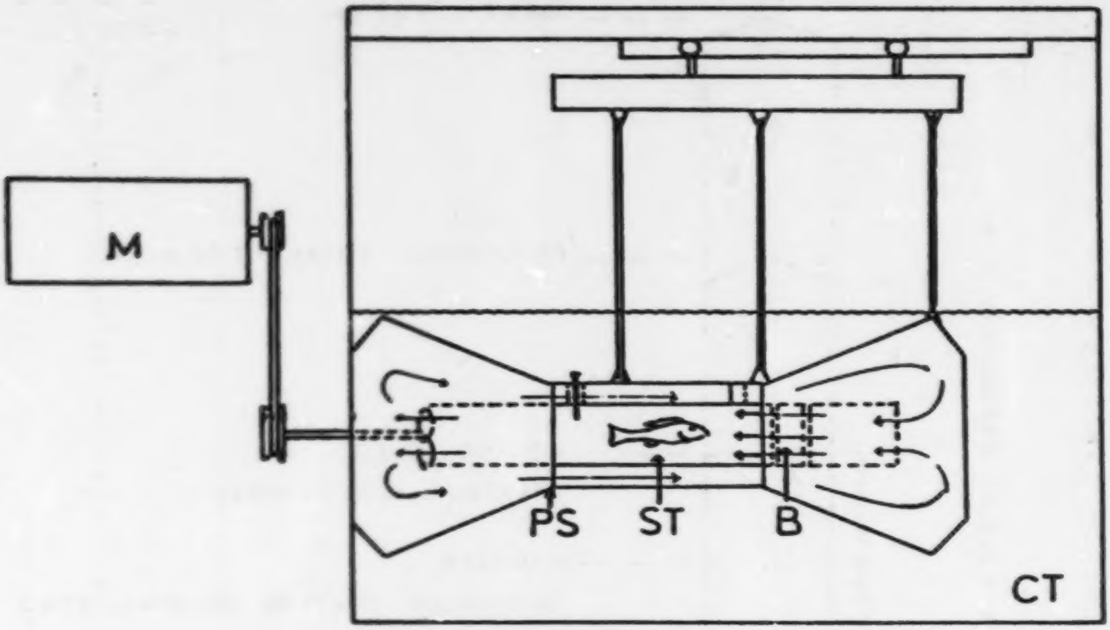

Fig. 2. Schematic diagram of 207-liter modified Blazka respirometer. M--variable speed, constant torque 10 h.p. motor; B--flow linearizing baffles; ST--transparent acrylic plastic swimming tunnel; PS--posterior screen; CT--constant temperature water bath. Note the trolley system for suspending the respiration chamber, which separates at the gasket between the swimming tunnel and the impeller portions. (Adapted from Wakeman and Wohlschlag 1978.)

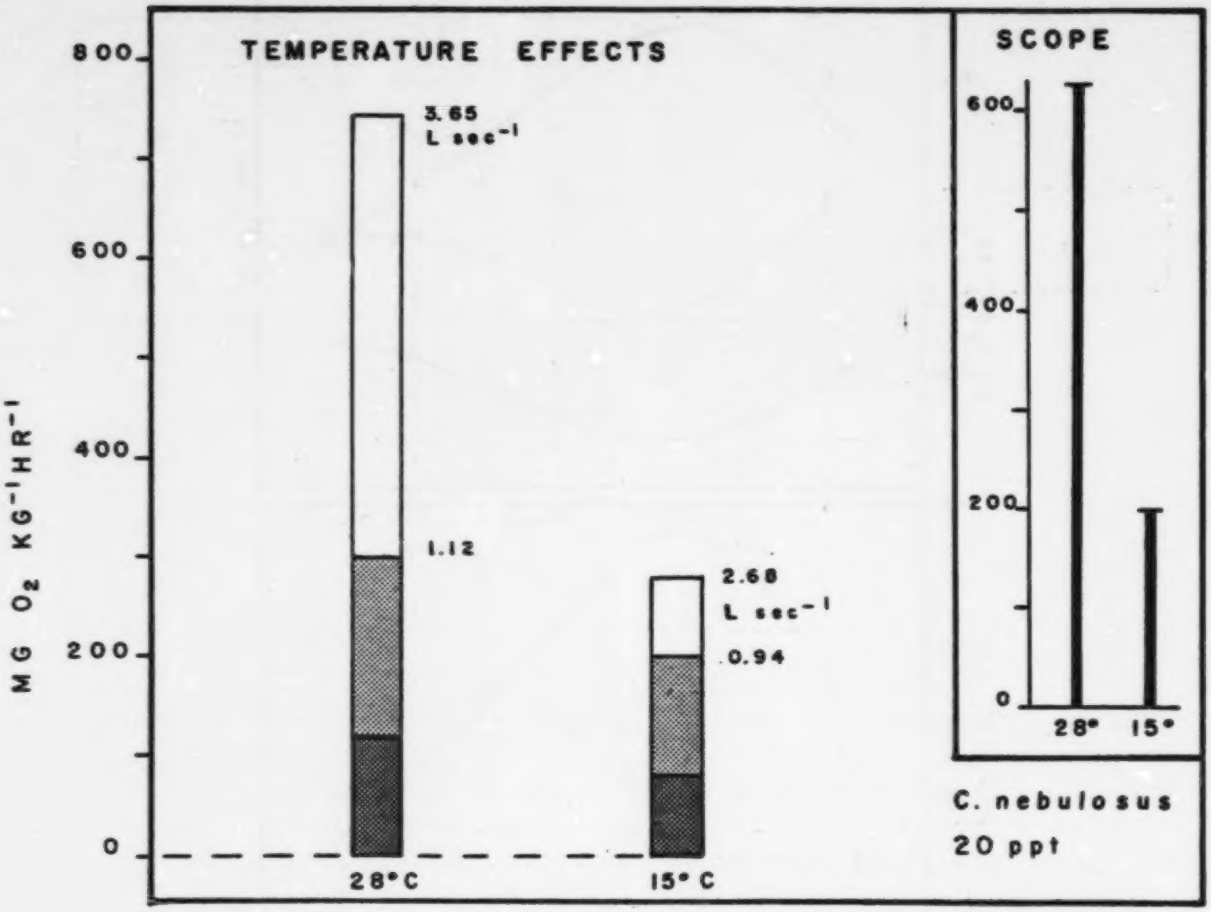

Fig. 3. Active, routine and standard metabolic levels, along with scope plots, at 28 and 15°C and at a salinity of 20 ppt for the spotted seatrout. The maximum sustained swimming speeds and the routine speeds are in lengths per second. After Wohlschlag (1977).

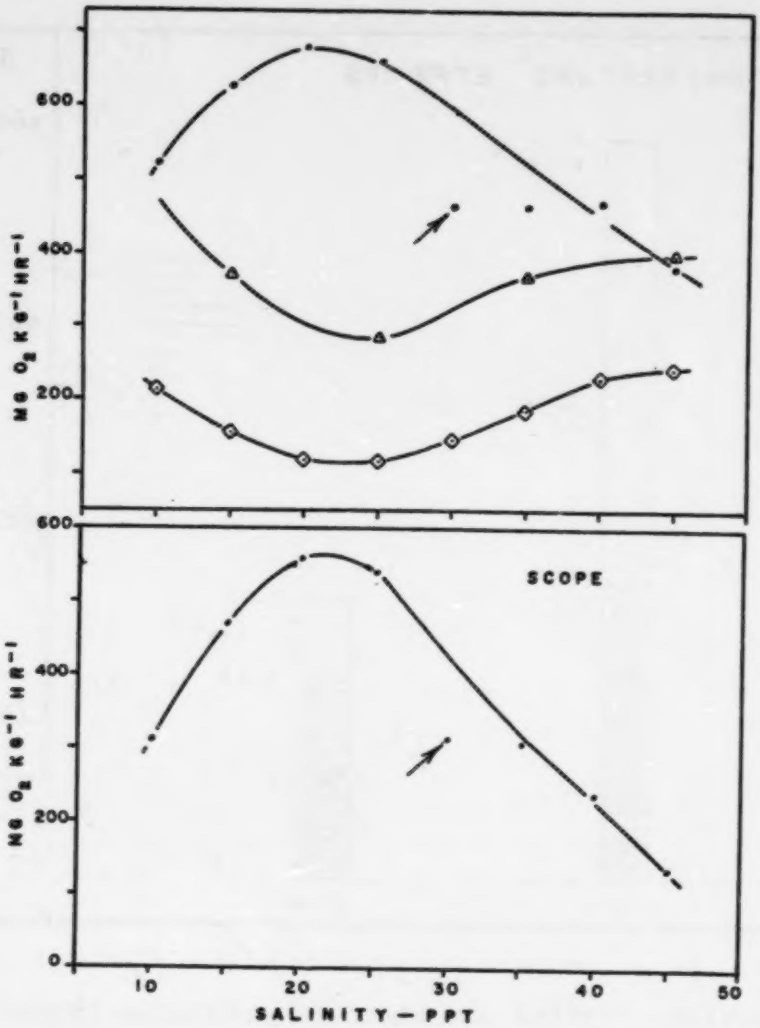

Fig. 4. Top panel--metabolic levels of the spotted seatrout at active (heavy points), routine (triangles), and standard (diamonds) conditions at 28°C and a range of salinities. Lower panel--metabolic scope for maximum sustained activity over a range of salinities at 28°C from data above. Arrows indicate depressed metabolic levels of active fish in poor condition, as illustrated in Fig. 6. After Wohlschlag and Wakeman (1978).

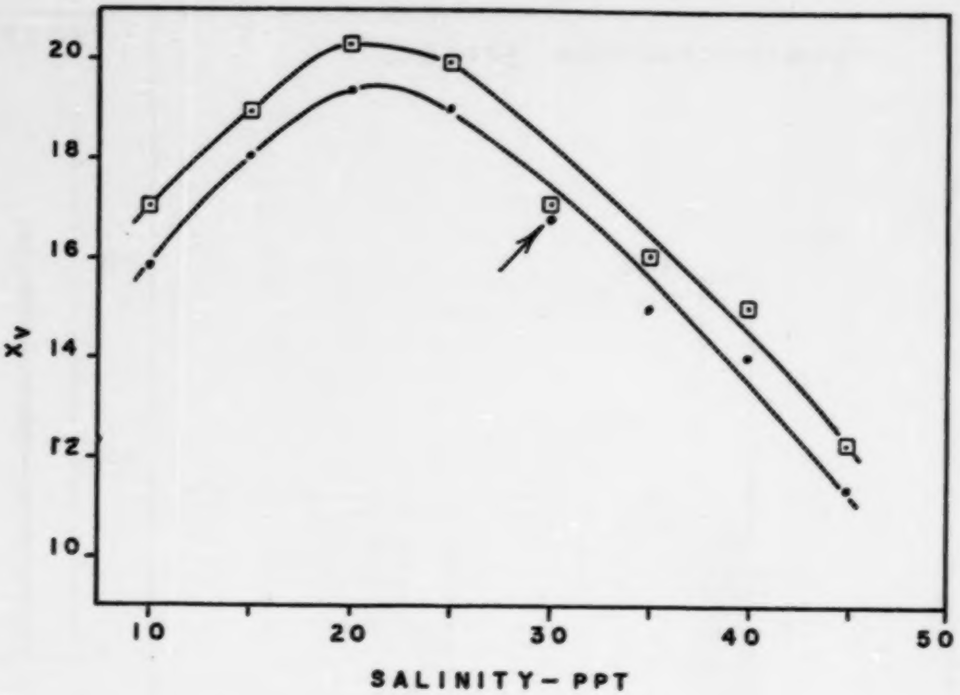

Fig. 5. Swimming characteristics of the spotted seatrout at 28°C and a range ot salinities. Swimming rate, X_v, expressed as square root of body length (cm) per second. Heavy points are averages of the maximum sustained (active) rates. Squares indicate the highest individual rates observed at each salinity. Arrow denotes fish in poor condition with depressed activity (see Fig. 6). After Wohlschlag and Wakeman (1978).

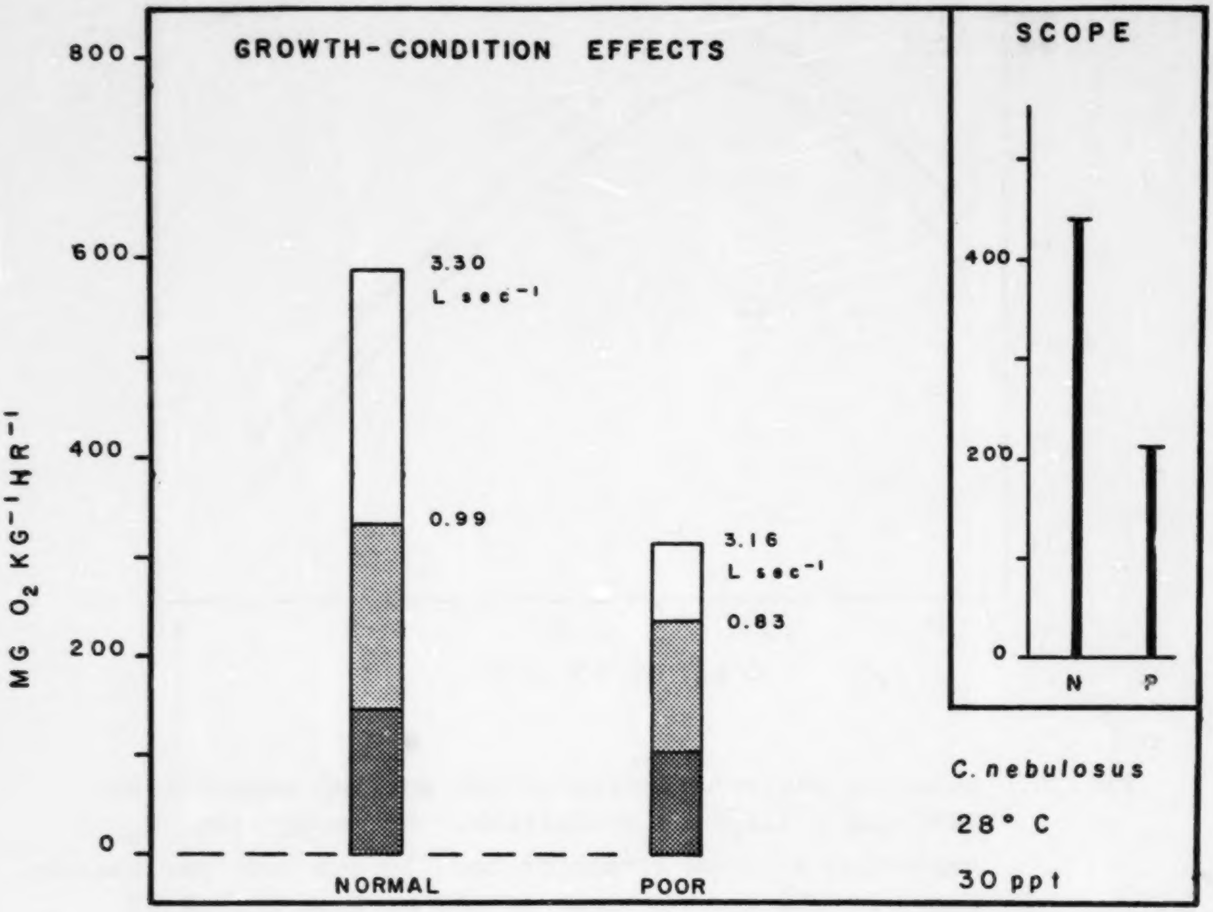

Fig. 6. Growth-condition effects on spotted seatrout at 28°C and 30 ppt salinity in late summer. Metabolic levels shown at active, routine and standard rates with accompanying swimming speeds for active and routine rates. Note that the scope for normal fish is greatly reduced for those in poor condition. After Wohlschlag (1977).

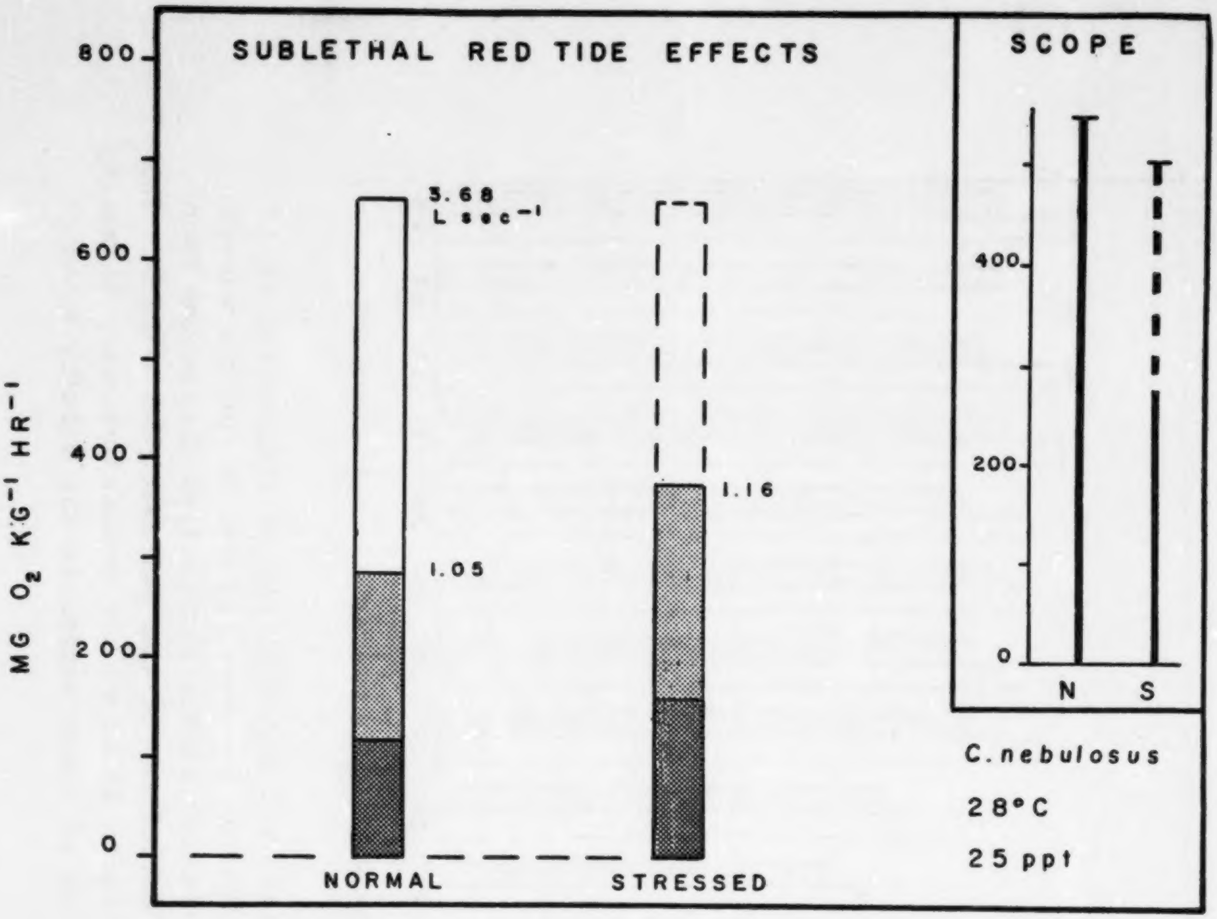

Fig. 7. Small red tide bloom effects on spotted seatrout metabolism at 28°C and 25 ppt salinity. Note that even if the active metabolic rate (which was not measured for the fish in waters exposed to bloom organisms) were not reduced, the scope is diminished because the standard rate of the exposed fish increases. After Wohlschlag (1977).

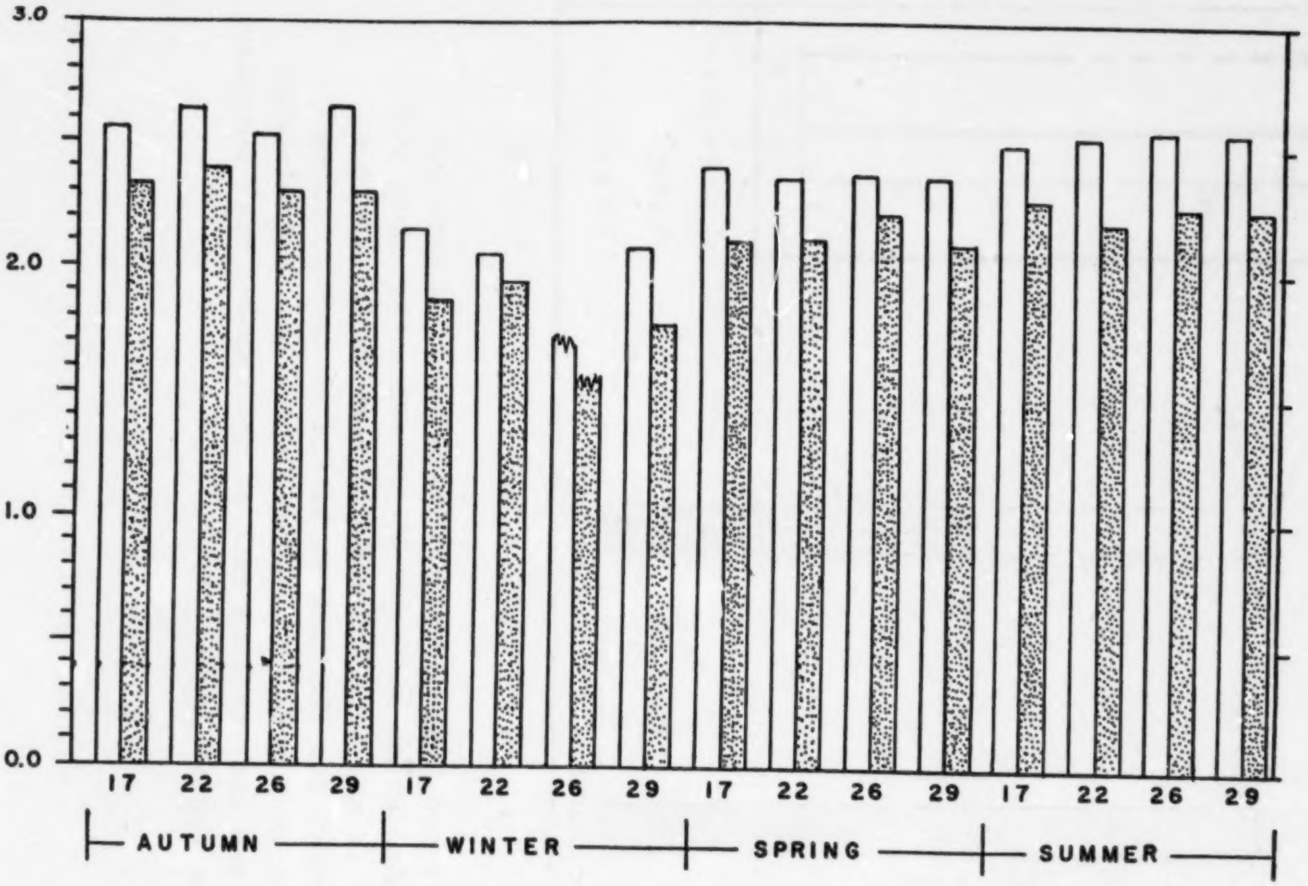

Fig. 8. Seasonal values of routine metabolic levels of striped mullet in waters from several Galveston Bay stations and in control waters. Note that the "clean" control waters caused less depression than the chronic, low level toxicities of the Galveston Bay waters. The incomplete plots for Station 26 in winter denote temporary lethality possibly due to an influx of toxic waters in the Trinity River. From Wohlschlag (1973).

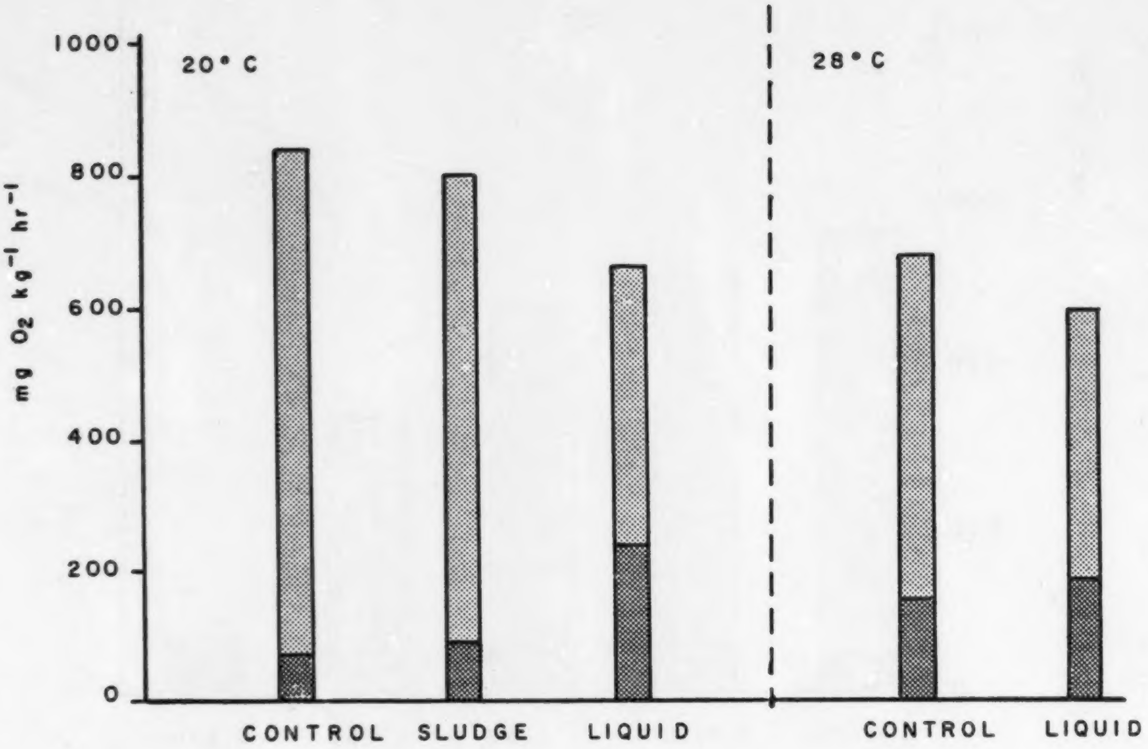

Fig. 9. Partially detoxified petrochemical sludge effects at a dilution of 0.2% v/v (about 1% of the LC_{50} level) on the red snapper both at 20°C with examples of separated sludge or of liquid portion only; at 28°C with liquid portion only. Scope in light shading; standard metabolism in dark shading.

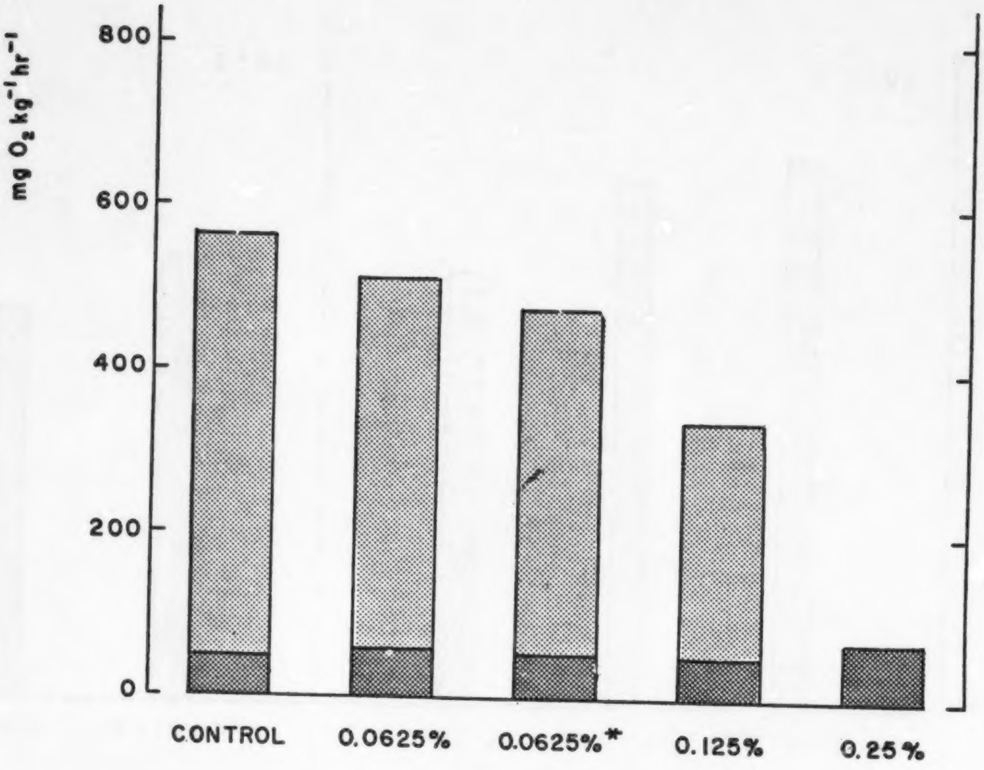

Fig. 10. Metabolic responses of spotted seatrout at 28°C and 35 ppt salinity to a partially decomposed composite Puerto Rican pharmaceutical industrial waste mixture at various dilutions. Asterisk denotes repeated test on mixture that had been shipped in glass instead of plastic containers. The lightly stippled area indicates metabolic scope; the darker stippled area indicates the standard metabolic rates. Note that at about 0.25% waste concentration the fish would maintain themselves at a standard rate for several days, but would suffer mortality if forced to swim. After Wohlschlag and Parker (MS).

	Control	Composite	Upjohn	Merck	Bristol	CAPRI	Squibb	Pfizer
Concentration (v/v %)	0.0	0.5	0.5	0.5	0.00625	0.5	0.5	0.5
Exposure time	0	1 hr	2 hr	25 min	2 hr	2 hr	110 min	2 hr
Average maximum swimming rate (Ls^{-1})	3.6	2.0	2.0	2.8	3.1	2.6	2.9	2.6

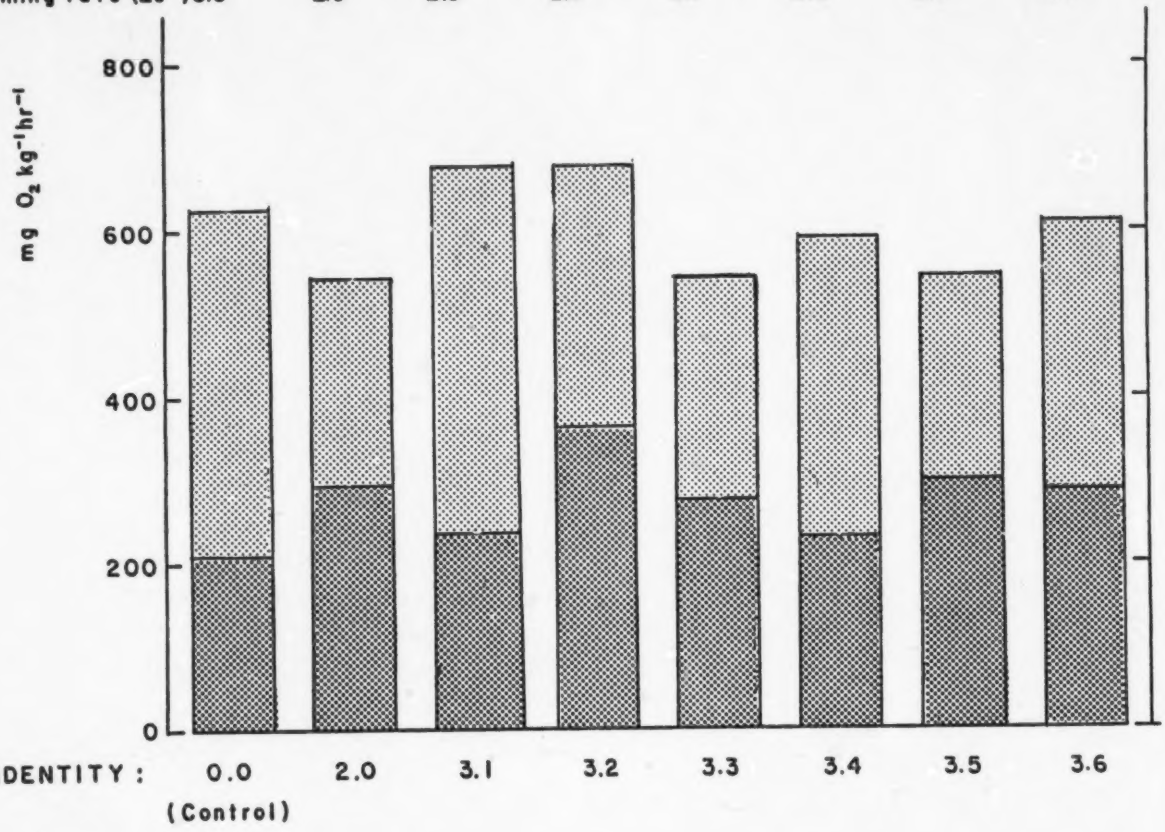

Fig. 11. Exposure-recovery experiments with spotted seatrout to pharmaceutical wastes at dilutions sufficient to cause visible stress at tabulated exposure times and concentrations. Data based on experiments at 28°C and 35 ppt salinity with metabolic rates for an average recovery time of about two days in clean sea water. Maximum sustained swimming rates remained depressed in all cases after about two days. Standard metabolic rates in heavy shading; scope for activity in light shading. After Wohlschlag and Parker (MS).

ENZYMES AND ANTIBODIES AS SENSORS OF TOXIC SUBSTANCES

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INTRODUCTION

Biodetection, a new adjunct to conventional analytical chemical techniques, is based on the application of biochemical systems to the detection and monitoring of toxic substances in the environment. It is an exciting approach to many analytical problems because it offers sensitivity, selectivity, low cost, and the opportunity for real-time monitoring of air and water for subtoxic levels of pollutants. This article describes the rationale behind the use of these enzymes and antibodies, and shows how they can be used to solve real problems in monitoring and detection of enzyme inhibitors and antigens in air, water, and soil.

Both insects and, unfortunately, humans are sensitive to poisoning by modern insecticides. The factor in both insects and humans that makes them sensitive to insecticides is an enzyme. Enzymes are used by nature to accelerate chemical changes in living things that ordinarily would occur too slowly. As biological catalysts, they are used over and over during metabolism and other chemical processes. The specific enzyme that is inhibited in this case, cholinesterase, is essential for proper transmission of impulses along nerve pathways. Blocking of this enzyme's function by reaction with pesticides interferes with proper nerve-impulse propagation and results in rigid muscular paralysis in the victim, whether man or insect.

Of all the thousands of enzymes required for function of the human body, only one, cholinesterase, is markedly affected by organophosphate and carbamate pesticides. Furthermore, a lowered blood level of this enzyme is very strong evidence that the person being tested has been exposed to these kinds of chemicals. The inhibition of cholinesterase is thus extremely specific for only a certain class of compounds (pesticides) and, among these, only the organophosphate and carbamate pesticides are effective as inhibitors.

It has proved possible to remove this enzyme from animals, purify it, and incorporate it into instrumentation designed to detect and quantify pesticides in the environment. This combination of electronics and material from living tissue for the estimation of compounds in nature has been pioneered by the Life Sciences Section of Midwest Research Institute. We have named the process "biodetection." The biological part of the technique provides rapid sensitivity and specificity, while the electronics portion magnifies the signal that arises from the biological "sensor" into handy readout formats such as chart recordings or digital displays.

Biodetection uses the remarkable specificity and sensitivity that are inherent in many biological systems. These systems have developed during aeons of

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biological evolution into processes that have an extremely high degree of selectivity toward their target molecules. Enzymes generally react with only one compound or substrate to cause its chemical transformation into a different compound. Antibodies, a primary defense against infection in animals, are very specific in their reaction with antigens. Other biological systems are notable for their specificities such as chemotaxis (the attraction of an organism to a chemical) and even the senses of taste and smell. Some of these processes are the subjects of intense experimentation in the laboratories of Midwest Research Institute for possible application in biodetection. Others are under consideration for future study.

USES OF CHOLINESTERASE

Of all these sensitive systems, enzymes are currently the most attractive, in part because of the ease of handling of many enzymes. Also, more is known about enzymes and how they work than about the more complex biological processes.

Of the thousand or so enzymes that have been isolated from biological tissues and characterized, cholinesterase is so far the most applicable for biodetection. One reason for this is that systems based on cholinesterase are sensitive to organophosphate and carbamate pesticides--the most widely used types of pesticides in the United States.

The enzyme has several attributes which make it attractive for use in an instrument. For example, cholinesterase is easily immobilized or secured onto a solid support such as glass beads or polyurethane foam. Thus, the same enzyme moelcules can be exposed to a water sample and, if pesticide inhibitors are not present in great concentrations, exposed again and again without substantial losses of activity. Without immobilization to a solid support or some other enzyme retention technique, the enzyme, which is not inexpensive, would be washed away after analysis of each sample. Over long periods of testing, the immobilized, reusable enzyme system can result in significant financial savings compared to enzyme-wasting systems. Cholinesterase is also relatively stable over considerable ranges of pH and temperature. This factor results in increased adaptability and ease of handling of the enzyme. In addition, there are several ways to measure the activity of the enzyme. This makes the enzyme extremely versatile for incorporation into instruments of varying design and complexity. For example, in the presence of the appropriate substrate and color-producing reagents, cholinesterase activity can be measured spectrophotometrically. When another substrate is used, the activity can be estimated by the rate of acid production by the enzyme. For field applications, the enzyme can be immobilized on paper discs fabricated so that a change of color or a failure to change color will alert a worker to the presence of dangerous pesticide concentrations. Finally, electrometric techniques have proven to be invaluable for real time or instantaneous and continuous monitoring of natural and workplace environments. Each of these measurement techniques has been incorporated into pesticide detectors.

Detection Principle--Enzymes are catalysts which increase the rates of specific reactions in living systems. Classically, the reaction of an enzyme with its substrate is given by

$$E + S \rightleftharpoons ES \rightarrow P + E \ ,$$

where E is the enzyme, S is the substrate, ES is the enzyme-substrate complex, and P is the product. Enzyme is reformed during the reaction and functions as a catalyst.

Formation of the product, under appropriate conditions proceeds linearly with time as in the "uninhibited" curve in figure 1.

For an enzyme such as cholinesterase, reaction with a pesticide inhibitor (I) results in the formation of an enzyme-inhibitor complex (EI) that is not functional.

$$E + I \longrightarrow EI$$

That is, the complex cannot make product.

Biological Amplification--If 25% of the enzyme molecules are inhibited, only 75% will be available to make product, so product will be formed at 75% of the uninhibited rate. This is shown diagrammatically in figure 1. Since a certain constant number of new product molecules are formed each minute, the sensitivity of the system to pesticides can be increased by allowing longer incubation times. For example, let us imagine that each cholinesterase molecule processed about 84,000 substrate molecules per minute. Inhibition of only one enzyme molecule by one molecule of pesticide would result in the formation of 84,000 fewer product molecules during the first minute of incubation. Theoretically, if the incubation were 4 min rather than 1 min, the inhibition of one enzyme molecule by one pesticide molecule would prevent the formation of 336,000 product molecules, with a resultant apparent signal amplification of 1:336,000. This phenomenal increase is due to the reaction kinetics of the enzyme and is exclusive of electronic amplification. It has therefore been given the name "biological amplification."

APPLICATIONS OF BIODETECTION USING CHOLINESTERASE

Early interest in the possible use of cholinesterase as a sensor for inhibitors in the environment came from the Department of Defense (DOD). Certain chemical agents called nerve gases are potent inhibitors of cholinesterase and can be lethal when encountered by humans. The DOD has maintained a continued interest in the detection of these compounds for the safety of personnel, not only in the field where chemical agents might have been deployed as a tactical weapon, but also in chemical demilitarization plants where stockpiles of nerve gases are being destroyed. The Army has supported research and development of instrumentation that ranges in sophistication from real-time monitors that feature preset alarm levels and time integration to simple paper discs which change color in the absence of inhibitors.

Much of the technology developed during these programs is directly applicable in today's pesticide industry. Real-time monitors developed for chemical-agent demilitarization plants can be used, occasionally without major modification, in commercial pesticide-manufacturing plants.

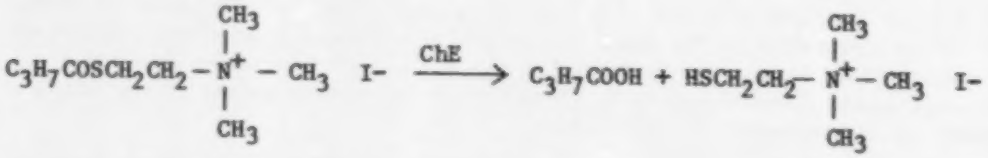
Many of the developments that were initiated during support from DOD contracts have been further refined with support form other nonmilitary agencies of the U.S. Government and private industry. Several of these areas of research have resulted in byproducts or spinoffs of prototype instruments with a wide range of capabilities. Several of the more important of these advancements are described in the following sections.

Real-Time Monitors--Many of the scenarios in which pesticides pose a potential health threat require real-time displays of pesticide levels. For example, in some areas in most pesticide manufacturing plants, accidental spillage can occur suddenly and with little visible indication. Workers in these areas need to be alerted as soon as possible. In another example, wastewater effluent from a pesticide plant can, at times, exceed pesticide levels considered safe by the Environmental Protection Agency (EPA). High pesticide concentrations here must be detected immediately so that the contaminated water can be shunted into reservoirs for further treatment to prevent dangerous pesticide levels from reaching wildlife and human drinking-water supplies.

Several prototype real-time monitors have been constructed to meet particular needs of portions of government and industry. The Immobilized Enzyme Alarm (IEA) was developed as a high volume air monitor for the U.S. Army. It passed severe environmental chamber tests and maintained a sensitivity of 0.0001 μg agent per liter of air. The Immobilized Enzyme Monitor (IEM) (fig. 2) is a direct descendant of the IEA. The IEM was designed for real-time monitoring in pesticide plants during industrial hygiene studies, although it is now being used continuously to insure worker safety. In 1976, this instrument won an IR-100 Award, which is given each year to the 100 most significant new technical products of the year by Industrial Research, Incorporated. The continuous Aqueous Monitor (CAM-1) was built during a program to detect low levels of organophosphates and carbamates in water, and received an IR-100 award in 1972 (fig. 3).

Each of these real-time monitors, although sampling from different media (air or water), functions by using very similar computer-controlled processes.

How They Work--As mentioned above, immobilized cholinesterase has been used for the concentration and detection of inhibitors in air and water. In order to determine the relative activity of the enzyme product before and after exposure to the potential enzyme inhibitors, a synthetic substrate for the enzyme was chosen that would be easily detectable by electrochemical means. In this case, butyrylthiocholine iodide (BuSChI) was chosen because the unhydrolized substrate was stable in the presence of platinum gray electrodes to which a constant current was applied, whereas this same substrate after hydrolysis by cholinesterase was readily oxidized electrochemically.



$$C_3H_7COSCH_2CH_2-\overset{\overset{CH_3}{|}}{\underset{\underset{CH_3}{|}}{N^+}}-CH_3\ \ I^- \xrightarrow{ChE} C_3H_7COOH + HSCH_2CH_2-\overset{\overset{CH_3}{|}}{\underset{\underset{CH_3}{|}}{N^+}}-CH_3\ \ I^-$$

In this reaction, a thioester is cleaved by cholinesterase (ChE) to give thiocholine iodide. Under the influence of an electric current a part of it is converted to the disulfide.

$$2\ RSH \xrightarrow{\text{anode reaction}} RS - SR + 2H^+ + 2e^-$$

Through the use of gel-entrapped butyryl cholinesterase on an open-pore polyurethane foam pad, it is possible to obtain good contact of the enzyme with the inhibitors or the substrate solution. The activity of the enzyme can be determined electrochemically by passing air and substrate through the enzyme pad, while 2 μa of current is applied to the electrodes in contact with the upper and and lower surfaces of the enzyme pad. If the enzyme is active, the substrate will be hydrolized as it passes down through the pad with the resulting presence of thiol at the lower platinum electrode (the anode) where it is sensed by the generation of a potential of ∿ 250 mv. On the other hand, if the enzyme is inhibited by an organophosphate or carbamate, the thiol is not formed and a potential of ∿ 500 mv is generated at the electrodes. Thus, by observing the changes in the cell potentials before and after exposure to enzyme inhibitors, it is possible to observe whether none, part, or all of the enzyme has been inhibited by the materials brought into contact with it. A simple electronic circuit can be used to signal an alarm when the voltage rise exceeds a preset threshold voltage.

In order to convert this electrochemical cell into the air or water monitor, it is necessary to establish a detection cycle and to add some components; these are shown in figure 4. The objective of the cycle is to introduce a time interval during which the enzyme pad is exposed to potential inhibitors (2 min) and an enzyme activity test period (1 min). Comparison of cell voltages from successive cycles is necessary to determine the presence or the absence of enzyme inhibitors. That is, increases in voltages from cycle to cycle are evidence for the presence of inhibitors; if the cycle-to-cycle increases are large, then the amount of inhibitor is also large. Before the activity of the enzyme pad is completely gone, it is necessary to add a fresh one.

Figure 4 shows the fluid pathways in the monitoring system and the sequence of events making up the detection cycle. For 2 min of each 3-min detection cycle, water (with or without inhibitors) is pumped through the enzyme pad. During the last 1 min of the detection cycle, air is blown through the pad at the same time that the substrate solution is introduced and a constant current is applied for the last 40 sec of the 3-min cycle. At this time, a voltage is generated that is characteristic of the amount of enzyme activity present on the enzyme pad. By comparison of cell voltages from successive cycles it is possible to determine whether enzyme activity has been lost due to inhibitors in the sample.

PASSIVE-DOSIMETRY

The Biodetection Group at Midwest Research Institute has had success in measuring personnel exposures using passive dosimeters. These dosimeters are termed passive since they depend on air currents and diffusion to bring airborne pesticides into their collecting matrices. Positive displacement samplers continuously aspirate local air through the collector but have the disadvantages that they require a power source to run a pump and are usually relatively bulk. The passive dosimeter, on the other hand, can be as simple as a thin collector medium (similar to filter paper), enclosed in a plastic badge with a hole to allow access of pesticide to the collector, and a spring clip to facilitate attachment to the worker's clothing.

After a predetermined time of wearing the badge (usually 8 hr), the medium is extracted with solvent to remove adsorbed pesticides. The extract can then be analyzed spectrophotometrically for cholinesterase inhibitors.

Spot Checker--A simple but effective device for detecting airborne pesticides in remote areas was developed and named the Spot Checker (fig. 5). It won an IR-100 Award in 1977. Each of the six orifices of the Spot Checker is covered with a filter impregnated with cholinesterase. During a single revolution of the hand crank, a preset volume (usually 25 ml) of air is drawn through each filter. If there are inhibitors in the air, they will combine with some of the immobilized enzyme and inactivate it. Subsequent addition of substrate and color reagent can be used as a test for remaining enzyme activity. Loss of activity indicates the presence of significant levels of pesticides in the air. Retention of activity shows that the air is relatively safe.

Soil Analyses--Another biodetection technique which has been developed and used extensively at Midwest Research Institute deals with the quantification of pesticides in soil. In the first step of this procedure, the pesticide was extracted from the soil by blending the sample in the presence of a water-alcohol mixture. The extract was then centrifuged at very high speed (15,000 rpm) to separate particulate matter. The second step was a manual spectrophotometric cholinesterase inhibition assay for actual quantification of the pesticide.

DEVELOPMENTAL PROGRAM FOR OCEAN MONITORING

It seems plausible that some of the toxic chemicals that are of concern environmentally would have an inhibitory effect on certain mammalian enzymes. Thus, it is possible that monitoring systems analogous to those described for cholinesterase could be designed and developed using other enzymes for the detection of other important pollutants. With this attractive prospect in mind, the National Oceanic and Atmospheric Administration is supporting work at Midwest Research Institute that has as its ultimate goal the development of ocean pollution monitors based on biochemical sensors.

The initial work of this program has been the identification of enzymes that are likely candidates as biosensors. Computerized searches of the scientific literature produced a lengthy list of enzymes that were reported to be inhibited by four classes of compounds on EPA's list of priority pollutants. These enzymes are shown in table 1. The numbers in parentheses under the name of the inhibitor class are the number of compounds in that class on the priority pollutant list. The numbers in the columns are the numbers of compounds in each class which were reported to have been tested for inhibition of the corresponding enzyme.

Other criteria were also used in the initial selection of enzymes. The enzyme had to be commercially available in order to avoid costly and time consuming isolation and purification procedures. Additionally, the enzyme had to be easy to assay and require inexpensive assay reagents.

From preliminary results given in table 2, it is clear that several of the enzymes are inhibited by specific compounds on the EPA priority pollutant list.

TABLE 1

ENZYME-INHIBITOR MATCHES FROM LITERATURE

Enzyme	Pesticides (16)	Phenols (11)	Metals (12)	Phthalates (4)
Lactic dehydrogenase[a]	4	5	6	0
Aldolase[a]	4	0	1	3
Hyaluronidase[a]	0	0	1	0
Glucose-6-phosphate dehydrogenase[a]	0	0	0	3
Malic dehydrogenase[b]	1	6	0	0
α-Amylase[b]	12	0	0	0
Pyruvate carboxylase[b]	5	0	0	0
α-Chymotrypsin[b]	0	3	0	0
Glucose oxidase[b]	0	0	7	0
Histidase[b]	0	0	6	0
Catalase	5	0	0	0
Alcohol dehydrogenase	1	3	4	0
Glyoxalate reductase	1	2	0	0
Glutathione-5-alkyltransferase	1	3	0	0
ATP Phosphoribosyltranferase	1	1	0	0
Hexokinase	1	4	0	3
Pyruvate kinase	1	2	2	3
Acetate kinase	1	2	0	0
Creatinine phosphokinase	1	2	0	0
Fructose-6-phosphate kinase	1	2	0	3
β-Amylase	12	0	0	0
Cholinesterase	3	0	0	0
ATPase	2	6	5	0
Carbonic anhydrase	1	0	7	0
Lipoxidase	0	1	0	0
Luciferase	0	1	5	0
Phosphorylase	0	3	0	0
Pepsin	0	1	0	0
Isocritric dehydrogenase	0	0	8	0
Glyceraldehyde-3-phosphate dehydrogenase	0	0	1	0
Dopamine-β-hydroxylase	0	0	1	0
Phosphoglucomutase	0	0	1	0
Rhodanese	0	0	3	0
RNA Polymerase	0	0	1	0
Alkaline phosphatase	0	0	1	0
Ureas	0	0	5	0
Fructose-1,6-disphosphatase	0	0	1	0
Trypsin	0	0	2	0
Carboxypeptidase	0	0	6	0

TABLE 1 (concluded)

Enzyme	Pesticides (16)	Phenols (11)	Metals (12)	Phthalates (4)
Invertase	0	0	1	0
Ribonuclease A	0	0	5	0
Urokinase	0	0	3	0
Lysozyme	0	0	1	0
3:5'-Cyclic nucleotide phosphodiesterase	0	0	1	0
L-Asparaginase	0	0	5	0
Creatinase	0	0	2	0
α-Galactosidase	0	0	2	0
Glucose-6-phosphatase	0	0	1	0
Pepsin	0	0	1	0
Cytochrome c reductase	0	0	0	3
Phosphoglucose isomerase	0	0	0	3
Enolase	0	0	0	3

---

a Enzyme chosen for initial laboratory work. One enzyme was chosen from each of the four classes of priority pollutants which have been identified. Also chosen for further literature search to find other known inhibitors.

b Enzyme chosen for further literature search to find other known inhibitors.

TABLE 2

PRELIMINARY RESULTS OF ENZYME INHIBITION TESTS

Enzyme	Inhibitor Class	Results[a]
Lactate dehydrogenase	Organochlorine pesticides	DDT, Toxaphene
Lactate dehydrogenase	Metals	Ag^{+}, Cd^{+2}, Cu^{+2}, Zn^{+2}, Se^{+4}, Hg^{+2}
Malate dehydrogenase	Phenols	4,6-Dinitro, 2,4,6-Trichloro, 4-Chloro-m-Cresol, Pentachloro
α-Chymotrypsin	Phenols	-
Aldolase	Organochlorine pesticides	-
α-Amylase	Organochlorine pesticides	-
Hyaluronidase	Metals	Cu^{+}, Se^{+4}, Hg^{+2}, Cn^{-}, Fe^{+3}
Glucose oxidase	Organochlorine pesticides Metals	
Histidase	Metals	Ni^{+2}, Cd^{+2},[c] Sb^{+3},[c] Pb^{+2}[c]
Glucose-6-phosphate dehydrogenase	Phthalates	
Catalase	Organochlorine pesticides	-
Urease	Metals	Cu^{+2}, Zn^{+2}, As^{+3}
Alcohol dehydrogenase (yeast)	Metals	
Alcohol dehydrogenase (liver)	Metals	
Hexokinase	Organochlorine pesticides	Aldrin, Chlordane, Heptachlor Epoxide, Endrin, DDT, DDD, DDE

---

[a] Blank indicates not yet tested. Dash indicates none of the tested compounds gave significant inhibition. Compounds and ions listed gave significant inhibiton (10^{-4} M).

[b] Not an EPA priority pollutant list.

[c] Possible activators.

These enzymes are good candidates for incorporation into continuous monitors. Work is underway to identify other enzymes that might be effective as biosensors. Future phases of this program will entail designing and fabricating real-time monitors based on these biosensors.

ENZYME AMPLIFIED IMMUNOASSAY

In addition to enzyme inhibition, antigen-antibody reactions are a sensitive and selective type of biochemical recognition reaction. Antibodies prepared in small animals to be sensitive to synthetic antigens have been used for several years for the quantitative analysis of drugs, metabolites, and hormones, most of which occur in extremely low concentrations. Most of these assays, however, rely on complex and bulky equipment, such as gamma and scintillation counters, to provide quantitation data about the antigen-antibody reaction. Work in our laboratories and elsewhere has shown that the detection of an antigen by an antibody can be signalled by a coupled enzyme system. The function of the enzyme is to amplify and provide a display, such as a color change, that is related to the amount of antigen present. We have initiated development of an enzyme-monitored immunoassay for the detection of 2,4-dinitrophenol (2,4-DNP), a toxic substance on EPA's list of priority pollutants.

Although there are a number of possible variations in the procedure, the various steps shown in figure 6 explain how the most attractive approach will function in the presence and absence of the pollutant to be detected.

Catalase is used to label the hapten, 2,4-DNP. Either nonlabeled 2,4-DNP to be assayed or catalase-labeled 2,4-DNP reacts with anti-DNP in solution. Then a fine, particulate, polymerized antibody to goat gamma globulin (the second antibody) contacts the solution and is separated out on a membrane or by a centrifugation step, depending on whether the test is being developed for field or laboratory use. The amount of catalase-labeled 2,4-DNP separated out with the second antibody is a function of the amount of unknown competing with this enzyme-hapten complex. Use of the substrate, hydrogen peroxide, and an oxygen electrode permits the quantitation of the unknown DNP.

BIODETECTION FUTURE BRIGHT

The use of biological mechanisms for the detection and quantification of compounds of interest is in its infancy. More and more frequently, articles are appearing in the analytical chemistry literature that describe new and practical uses of biodetection. Researchers are realizing that many biological systems have extraordinary chemical specificity and sensitivity. The challenge is to adapt these natural sensors to instrumentation and devices in ways that improve current methods of detection and analysis.

The future for the Biodetection Group at MRI is exciting since new discoveries in enzymology, immunology, and other areas of biochemistry are routinely revealing new biological mechanisms that may be applicable in biodetection. The future in this area of analytical chemistry looks very promising.

ACKNOWLEDGMENTS

Much of the work described in this article has been supported financially by the U.S. Army, the U.S. Environmental Protection Agency, and the National Oceanic and Atmospheric Administration. The author is grateful for the guidance given by Mr. William B. Jacobs and Dr. Louis H. Goodson and for the tireless laboratory effort rendered by the technical staff of the Life Sciences Section.

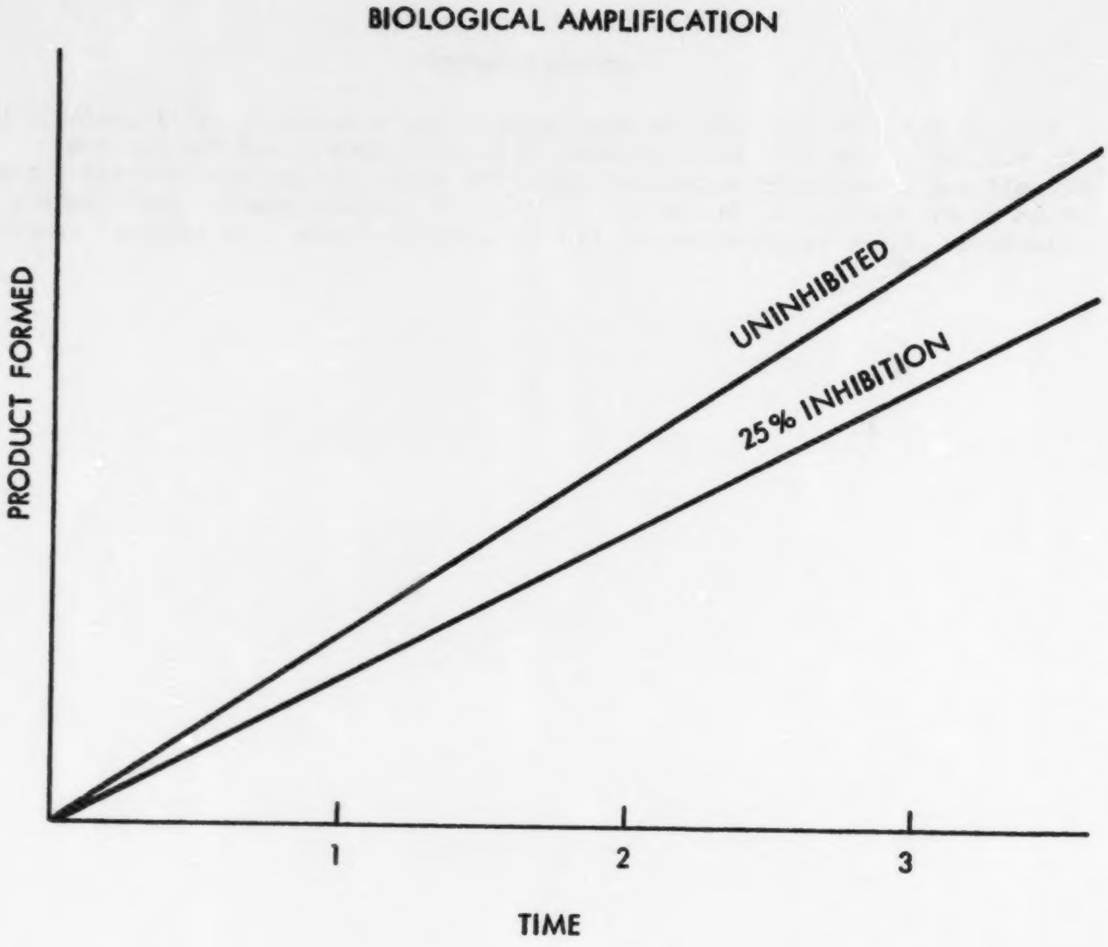


Figure 1 - Biological Amplification.

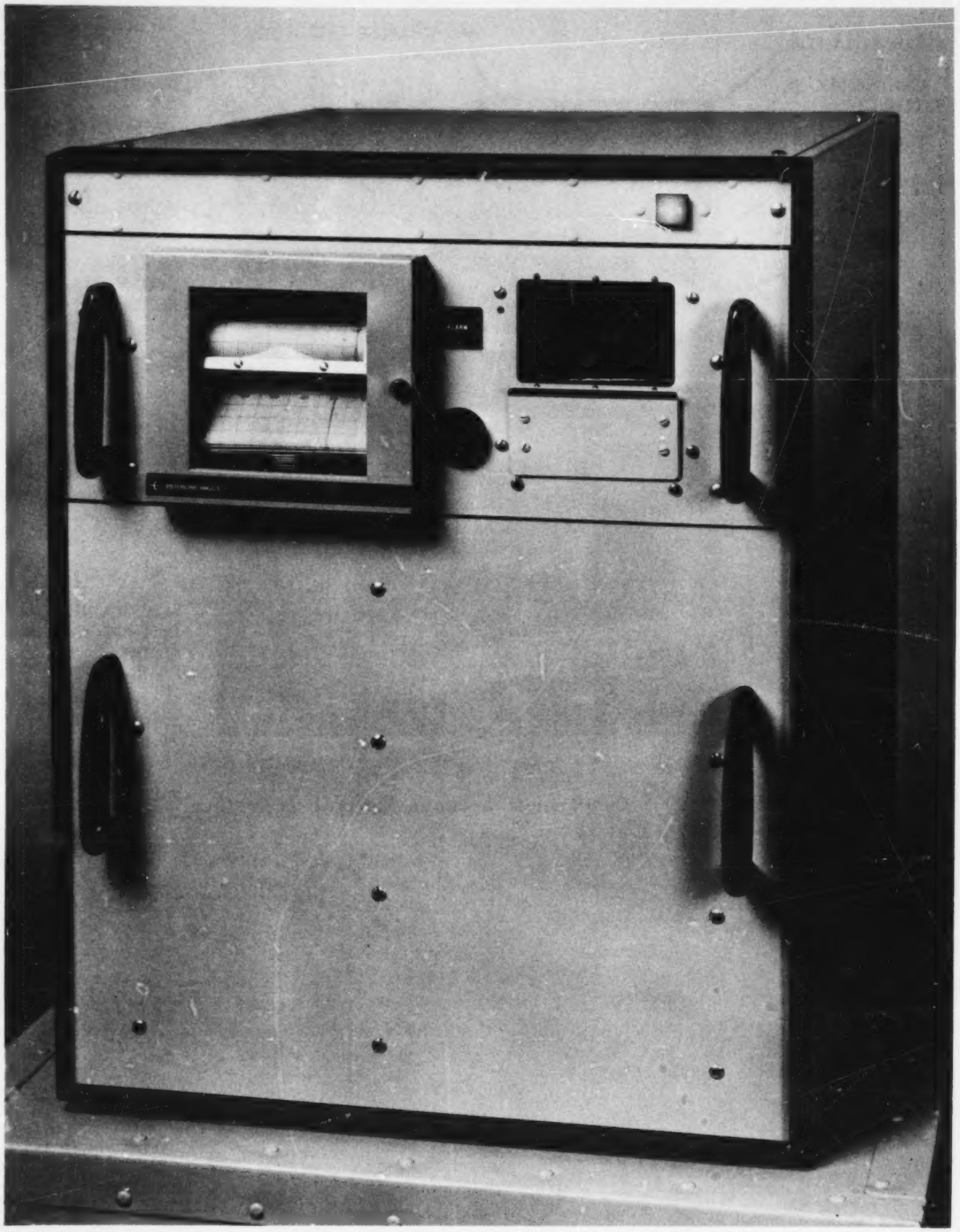


Figure 2 - Immobilized Enzyme Monitor

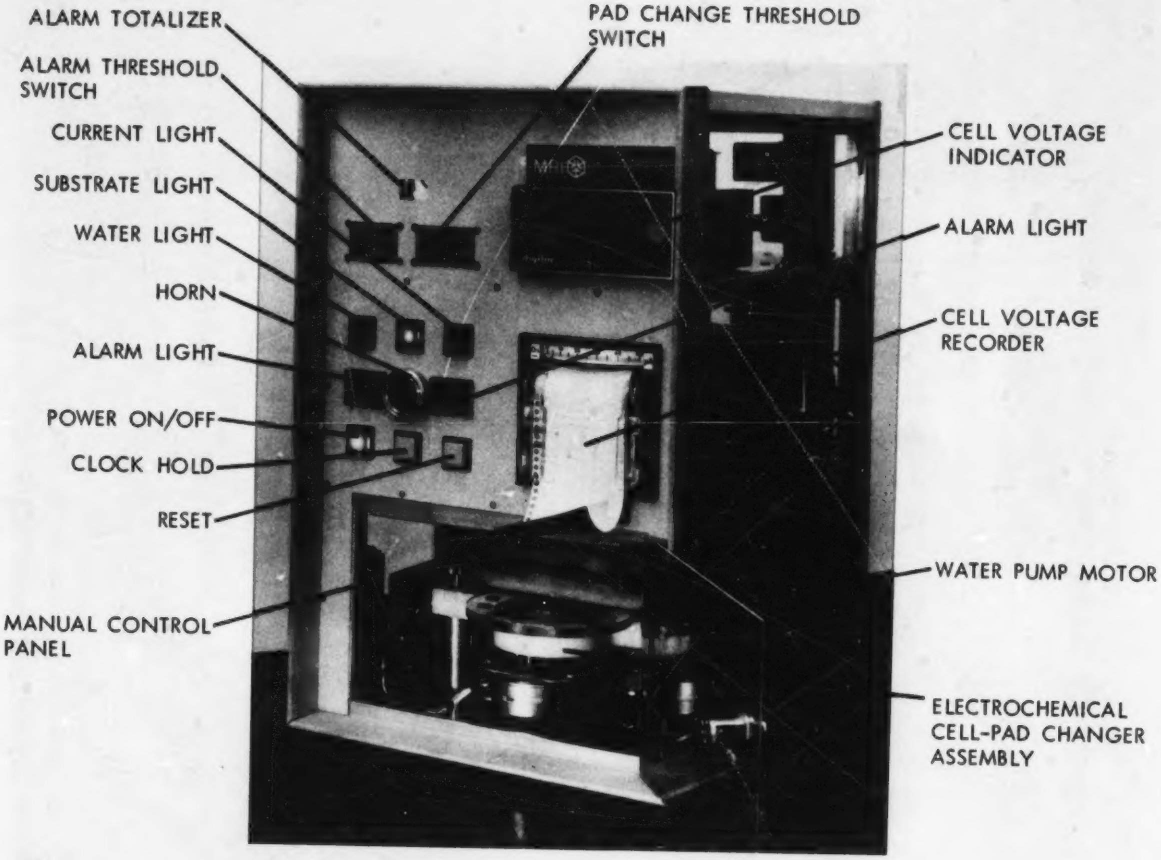

ALARM TOTALIZER
ALARM THRESHOLD SWITCH
CURRENT LIGHT
SUBSTRATE LIGHT
WATER LIGHT
HORN
ALARM LIGHT
POWER ON/OFF
CLOCK HOLD
RESET
MANUAL CONTROL PANEL
PAD CHANGE THRESHOLD SWITCH
CELL VOLTAGE INDICATOR
ALARM LIGHT
CELL VOLTAGE RECORDER
WATER PUMP MOTOR
ELECTROCHEMICAL CELL-PAD CHANGER ASSEMBLY

Figure 3 - Continuous Aqueous Monitor (CAM-1)

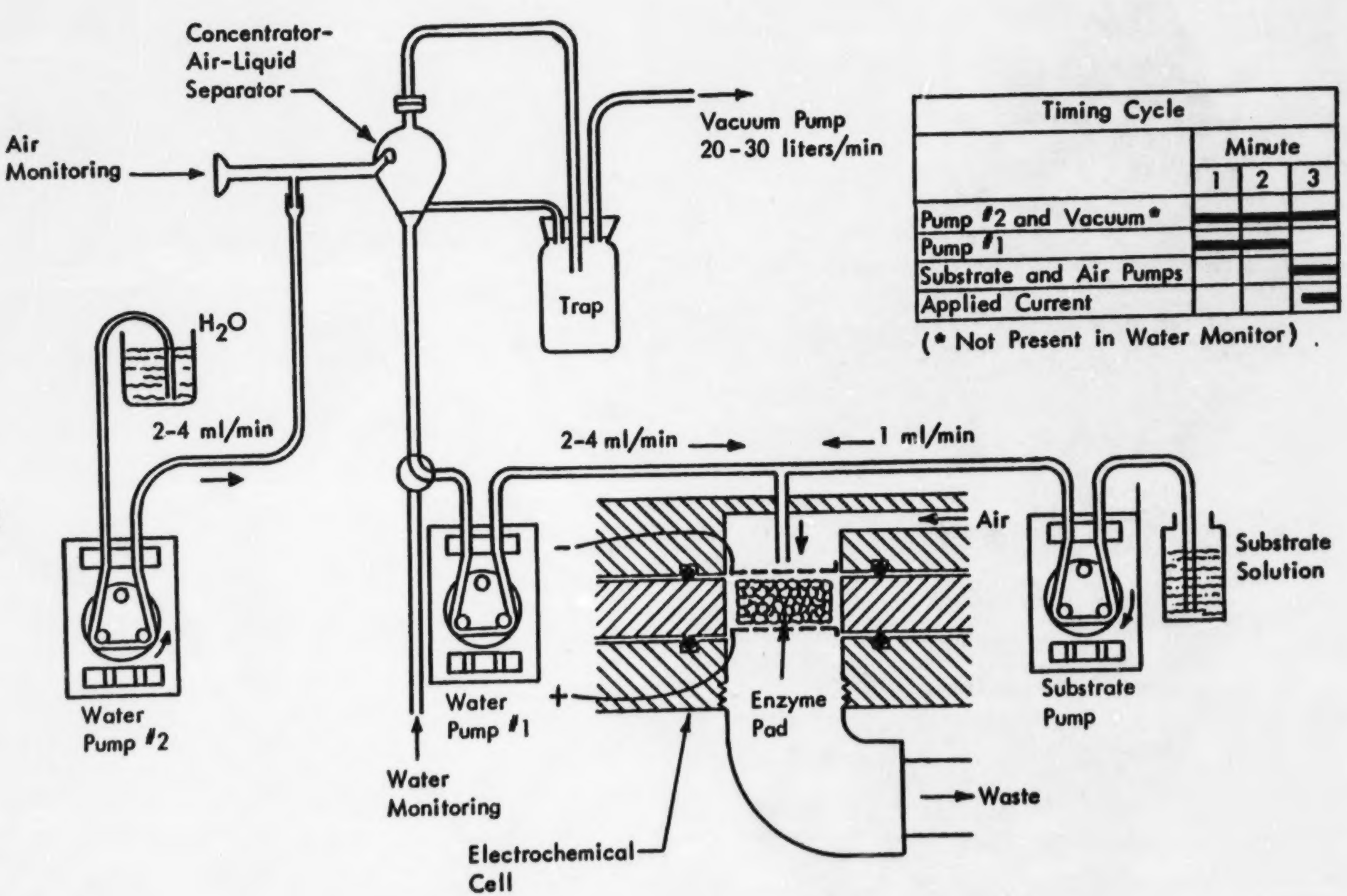

Concentrator-
Air-Liquid
Separator
Air
Monitoring
Vacuum Pump
20 - 30 liters/min
Trap
H_2O
2-4 ml/min
2-4 ml/min
1 ml/min
Air
Substrate
Solution
Water
Pump #2
Water
Pump #1
Water
Monitoring
Enzyme
Pad
Substrate
Pump
Waste
Electrochemical
Cell
Timing Cycle
Minute
1
2
3
Pump #2 and Vacuum*
Pump #1
Substrate and Air Pumps
Applied Current
(* Not Present in Water Monitor) .

Figure 4 - Flow diagram of air and water real-time monitors.
(The concentrator assembly in the upper left
is not used for water monitoring.)

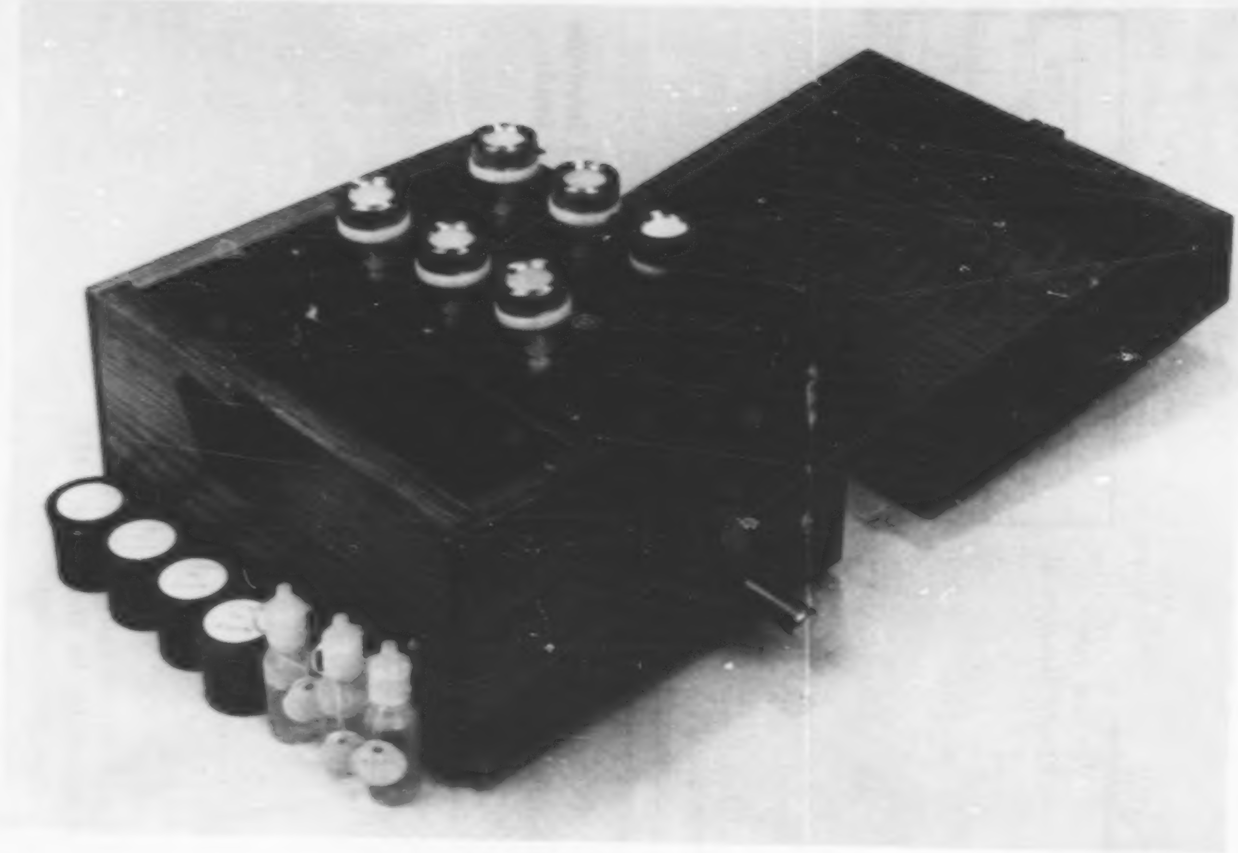


Figure 5 - Spot Checker

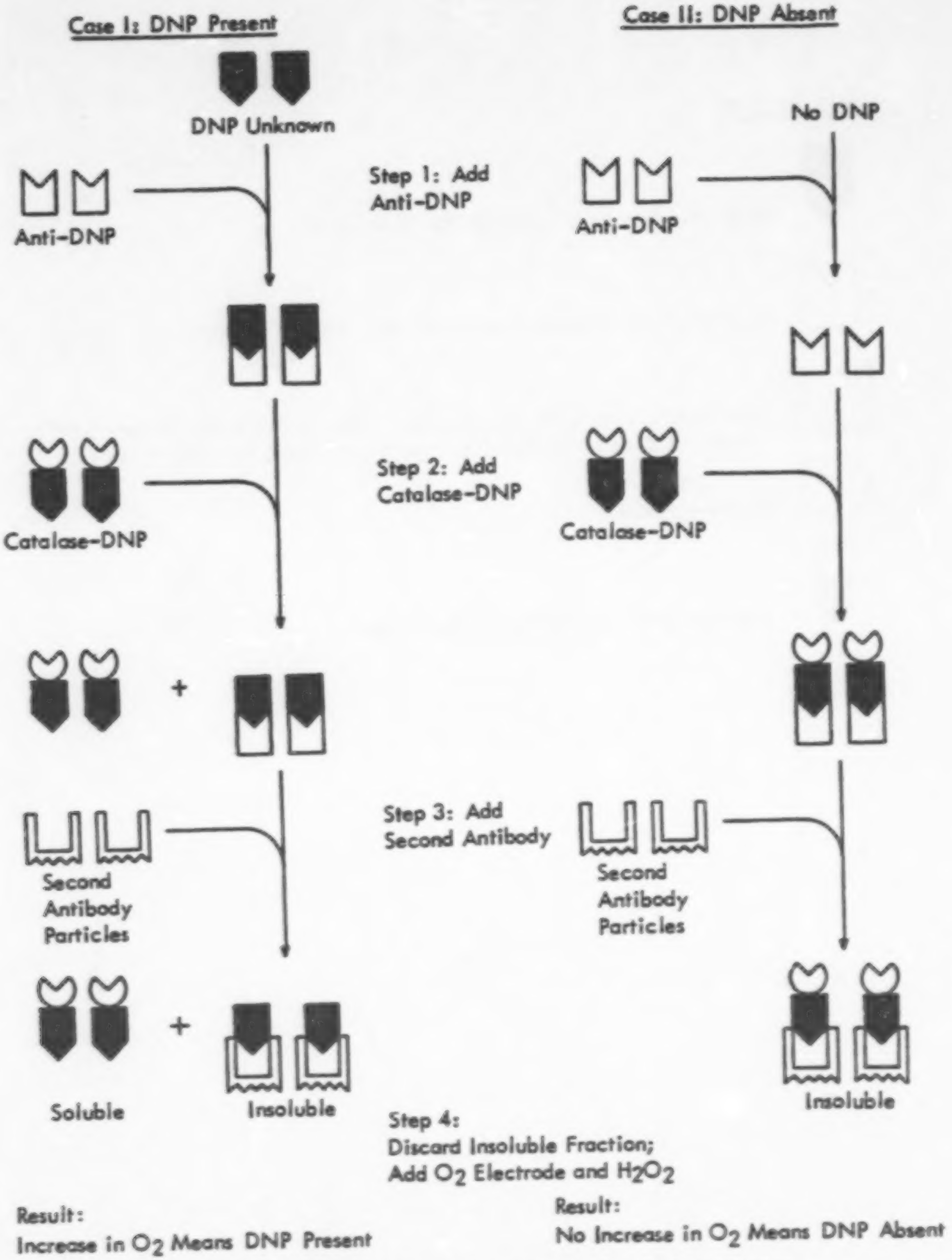
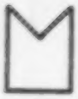

Case I: DNP Present
Case II: DNP Absent
DNP Unknown
No DNP
Step 1: Add
Anti-DNP
Anti-DNP
Anti-DNP
Step 2: Add
Catalase-DNP
Catalase-DNP
Catalase-DNP
+
Step 3: Add
Second Antibody
Second
Antibody
Particles
Second
Antibody
Particles
Soluble
+
Insoluble
Insoluble
Step 4:
Discard Insoluble Fraction;
Add O2 Electrode and H2O2
Result:
Increase in O2 Means DNP Present
Result:
No Increase in O2 Means DNP Absent

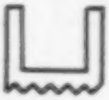
Figure 6 - Diagram of Enzyme Immunoassay for DNP Detection
(see next page for reagent code)

REAGENT CODE

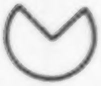
DNP, the "unknown" pollutant to be detected



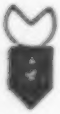
Anti-DNP, an antibody that binds DNP, made in rabbits



Anti-rabbit gamma globulin, a second antibody that binds the anti-DNP, made in goats, chemically treated to make it insoluble



Catalase enzyme



Catalase-DNP, covalently bonded reagent

BIOPROBES FOR THE MONITORING OF TOXIC COMPOUNDS AND HAZARDOUS MATERIALS IN WATER

by

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INTRODUCTION

The bioanalytical research group at the University of New Orleans is composed of biochemist and analytical chemists under the supervision of Dr. George G. Guilbault. The primary research interest has been recently devoted to the development of bioprobes for the analysis of substances of clinical, agricultural, and environmental importance. The so-called "bioprobe" is, in general, a device consisting of a sensor (i.e., an electrode) and bioactive materials (i.e., enzymes or antibodies) for highly selective analysis of certain compounds. During the past decade, we have successfully induced, isolated, and purified many enzymes. With the use of these enzymes, new bioprobes and analytical systems have been set up and fabricated for fast, simple, accurate, selective, and sensitive assay of a variety of compounds of interest. In this paper, only those analytical systems pertaining to pollutants present in water systems are described.

ANALYTICAL SYSTEMS FOR NITRATE AND NITRITE

It is well-known that the toxicity of nitrite is due mostly to the formation of carcinogenic N-Nitroso-compounds by the reaction between nitrite and secondary or tertiary amines in the animal tissues, and to the induction of methemoglobinemia (caused by nitrite oxidation of hemoglobin from the ferrous to the fernic form). Though nitrate itself is not toxic, it can be converted to nitrite inside an animal body by microorganisms present in the digestive tract. Due to the increasing use of nitrate and nitrite in foods, fertilizers, detergents, and other fields of industry nowadays, the development of fast, simple, accurate, and economical methods for regulatory analysis of nitrate and nitrite in portable water is urgently needed. Though numerous methods for NO_3^- and NO_2^- assay have been developed and reported elsewhere, most of these methods are either nonspecific and subject to interferences from diverse substances or very complicated to manipulate. The use of a bioprobe which combines speed, selectivity, and simplicity of operation for chemical analysis appears to be the best approach.

The development of nitrate and nitrite bioprobes is based on the following reactions:

$$NO_2^- + 6e^- + 8H^+ \xrightarrow[\text{reductase}]{\text{nitrite}} NH_4^+ + 2H_2O \qquad (1)$$

The nitrite is selectively and quantitatively reduced to an ammonium ion by nitrite reductase in the presence of electron donors, for example, reduced methyl viologen NADH, and the ammonium ion generated is sensed by an ammonium ion selective electrode.

$$NO_3^- + 2e^- + 2H^+ \xrightarrow[\text{reductase}]{\text{nitrate}} NO_2^- + H_2O \qquad (2)$$

As shown in equation 2, an enzyme, nitrate reductase, catalyzes the reduction of NO_3^- to NO_2^- which is in turn reduced to ammonium ion by nitrite reductase.

Source of Enzymes--Nitrate and nitrite reductases are generally found in certain microorganisms and plants and exist as constitutive enzymes in the living system. These enzymes can be induced as the growth conditions are altered. We have successfully induced, isolated, and purified severl nitrate reductases from E. coli and Chlorella vulgaris, and nitrite reductases from E. coli, Azotobacter chroococcum and spinach (refs. 1, 2, 3, 4, and 5). These enzymes differ from one another in their stability, specificity, and catalytic activity and especially in the ability to use a lower redox potential electron donor. In general, inducible nitrate and nitrite reductases are not as stable as constitutive enzymes during purification, immobilization, and storage but have higher specific activities.

Both stability and catalytic activity are important for the construction of a useful, sensitive, and fast response bioprobe. Thus, the use and selection of a proper enzyme for bioprobe depends mostly on the systems to be used for the analysis of the desired substrate.

Electrodes

a. Ammonia sensor - Several devices for sensing ammonium ion generated during enzymatic reaction have been developed in our lab and used for the construction of bioprobes as shown below.

b. Nonactin membrane electrode - Details on the preparation and operation of this type electrode have been described (ref. 6) and will not be mentioned here. Nevertheless, we like to point out that this electrode is subject to some K^+ and Na^+ interferences, thus limiting its application for selective analysis of NH_4^+.

c. Air Gap Electrode - To eliminate the cation interferences mentioned above, we used an air gap electrode to measure the amount of gas liberated from the enzyme - substrate reaction. An air gap electrode is composed of a combined pH electrode housed inside a polystylene cylinder and an air tight chamber. The gas evolved from the reaction mixture in the chamber diffuses to the electrode surface where it reacts with a wetting solution, thus causing a change of hydrogen ion concentration. The pH observed is proportional to the log concentration of the gas and common interfering ions, e.g., K^+ and Na^+, do not interfere.

d. Gas Selective Membrane Electrode - Ammonia selective gas membrane electrodes are now available commercially. For the detection of NH_3, these electrodes require a pH which is much higher than the optimal pH for most of the enzyme reactions. Therefore, it is not suitable for use as a bioprobe sensor. An ammonia gas sensing electrode which could detect the ammonium ion at lower pH's was then developed in this laboratory (ref. 7). The sensor was made by placing a piece of gas selective membrane over the surface of the radiometer pH electrode. A rubber plastic sealant and an "O" ring were used to fix the membrane and to prevent the leakage from the internal solution to the sample. This electrode gives a fast response time as well as a speedy recovery time and exhibits excellent performance in aqueous solutions without any interference originating from K^+, Na^+, or other cations. It is ideal for use in constructing a bioprobe for batch or continuous measurement of ammonia in solution.

3. Instrumental Setup - With the use of either soluble or immobilized enzymes, different systems have been set up for selective analysis of NO_3^- and NO_2^- in food as well as in water (refs. 2, 3, 4, 5, and 6). As described in these papers, soluble enzymes were employed for batch measurements and immobilized enzymes for applications in continuous flow systems. The ammonia generated was quantitated by an air-gap electrode. The only disadvantage in using this set up is that NaOH must be added to the chamber to liberate NH_3 from the reaction mixture for better response. A very simple bioprobe could be developed using a gas membrane electrode as a sensor for convenient, sensitive, selective, and direct analysis of NO_3^- and NO_2^- if the isolation of more active and more stable nitrate and nitrite reductases could be achieved. Research along this line is now in progress.

ANALYTICAL SYSTEMS FOR CYANIDE

Cyanide has been one of the toxic chemicals discharged in industrial effluents. It is especially abundant around iron and steel industrial areas. Several years ago, biological detection and decontamination systems were developed in our laboratory to monitor and remove this highly toxic pollutant from the environment.

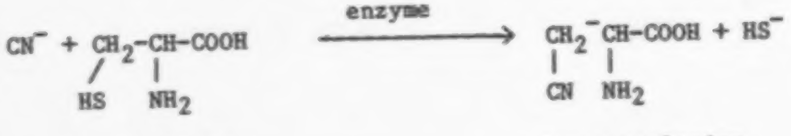
Source of Enzymes - Two enzymes, rhodanese and β-cyanoalanine synthase, have been isolated and used for this purpose (refs. 8 and 9).

a. Rhodanese - This enzyme was isolated and purified from the liver of higher animals and catalyzes the following reaction:

$$CN^- + S_2O_3 \xrightarrow{\text{enzyme}} CN^- + SO_3^{--} \qquad (3)$$

Since the products are not toxic, this enzyme is excellent to be used for decontamination purpose to remove CN^- from water.

b. β-Cyanoalanine synthase - This enzyme was induced from E. coli. 12, using a cultural medium containing 10^{-5} MKCN for growth. The purified enzyme catalyzes the injection of CN^- into the cysteine molecule with release of HS^-:



$$CN^- + \underset{HS}{CH_2}-\underset{NH_2}{CH}-COOH \xrightarrow{\text{enzyme}} \underset{CN}{CH_2^-}\underset{NH_2}{CH}-COOH + HS^-$$

cysteine

β-cyanoalanine

Because one of the end products, β-cyanoalinine, is a neurotoxin, this enzyme cannot be used for decontamination. However, the other end product, HS^-, is very useful as an indicating ion for selective monitoring of the CN^- content in water.

Sensors - Both cyanide and sulfide electrodes have been employed to monitor the cyanide.

a. Cyanide Electrode - The electrode was prepared from a plexiglass tube onto which a cyanide sensitive membrane was fitted. A silver wire in 10^{-2}M $AgNO_3$ solution was used as the internal reference (ref. 8). Nevertheless, this electrode is subject to interference from thiocyanate and sulfite ion.

b. Sulfide Electrode - The commercial sulfide electrode requires rather high pH for sensitive detection and is not proper for use as a bioprobe. A sulfide electrode which senses HS^- at lower pHs was made in the laboratory by gluing the compressed disc onto a glass tube using a silver billet electrode in 0.1M $AgNO_3$ solution as the inner reference system. This electrode is extremely selective when coupled with β-cyanoalanine synthase, constituting a cyanide bioprobe (ref. 10).

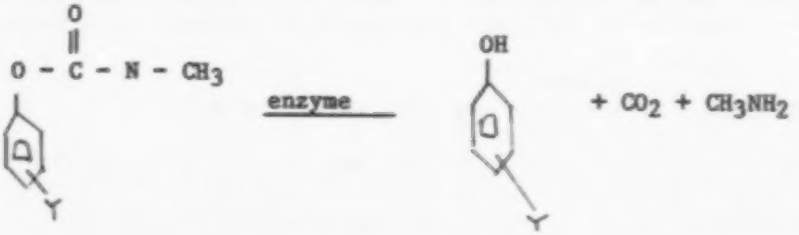
Instrumental setup - Both soluble and immobilized enzymes have been used in either batch or flow system for the measurement of cyanide ion in water system. The rhodanese bioprobe provides a sensitive and accurate means for CN^- analysis when compared with spectrophotometric procedures, since it requires less sample preparation and is easily adapted to an automated analyzer. Interferences from thiocyanate makes it less attractive for selective analysis.

The β-cyanoalanine bioprobe has been successfully used for batch CN^- assay, as well as for continuous monitoring of CN^- removal from water system. This probe exhibits high selectivity; no other ion interferences.

ANALYTICAL SYSTEMS FOR CARBAMATE PESTICIDES

During the past decade, carbamate pesticides have been widely used in nature and, hence, pose an ecological problem after use. Several years ago, studies were conducted and biological systems developed for the monitoring and decontamination of these pollutants using microbial enzymes as catalysts.

Source of Enzyme - An enzyme aryl acylamidase was isolated from pseudomonas striata that grew in mineral medium with acetanilide as the sole carbon and nitrogen sources for growth (ref. 11). The purified enzyme was found to be active toward the hydrolysis of phenyl or methyl carbamate pesticides.



(5)

Sensor - Based on the air gap sensor principle, a simple electrode was developed to measure the methylamine generated from the reaction (ref. 12). Methylamine concentrations ranging from 10^{-5} - 10^{-2}M can be easily detected. This electrode is selective and only slightly subject to dimethyl amine or ammonium ion interferences. It is ideal for the construction of a methyl carbamate bioprobe.

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POTENTIOMETRIC BIOSENSORS BASED ON HETEROGENEOUS CATALYTIC SYSTEMS

by

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Potentiometric ion-selective membrane electrodes are now routinely employed in many biological and environmental analyses. The advantages of membrane electrode-based methods include low cost, simple instrumentation requirements, ability to do in-situ measurements (i.e., sample turbidity and color are not a problem), and in-field capability. The range of substances measurable by these electrode systems can be expanded by coupling appropriate enzyme reagents to given electrodes to form selective enzyme electrodes (1). More recently, a new approach has been to use intact organelles and whole cells (i.e., heterogeneous biocatalysts) in conjunction with membrane probes for the purpose of biochemical analysis (2-8). In this report, the work done to date in this new area will be summarized and some speculation as to the future analytical possibilities using these heterogeneous systems will be presented.

In considering any biocatalytic process, a simplified expression of the reaction taking place can be summarized as

$$A \xrightarrow{\text{biocatalyst}} B$$

If product B is detectable by one of the many selective membrane electrodes available, it is a simple task to develop an electrode-based analytical method for substrate A. Previously, it has been assumed that purified enzymes were the most suitable reagents for such purposes, since the isolated enzymes should exhibit maximum biological activity and selectivity. However, it has recently been shown that in many instances intact cells or organelles can serve as the biocatalyst without loss of desired selectivity. This use of heterogeneous biocatalytic reagents in conjunction with membrane electrodes is possible because products of extraneous catalytic pathways present are not sensed by the selective potentiometric detector. Moreover, in cases where biological activity is dependent on the integrity of cell structure, membrane probes offer the simplest means of incorporating these particulate biosystems into practical analytical methods.

Table I lists the advantages of using such catalysts for analytical purposes. Table II summarizes the types of catalysts which may be used with membrane electrodes and the variety of analyses which have been or may in the future be performed.

An example of the use of intact organelles for selective analysis can be shown in the measurement of thyroxine (T_4) using an iodide selective membrane electrode and a rat-liver microsomal reagent (2). The microsomes catalytically deiodinate the T_4 to T_3 (triiodothyronine). The amount of iodide released can be directly monitored in the microsome suspension with the iodide probe. Figure 1 illustrates the analytical results and selectivity obtained using such a system.

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Table I.--Analytical advantages of heterogeneous biocatalysts

1) No need for costly isolation and purification of active component - low cost
2) Necessary cofactors, etc. are already present
3) Stability of catalytic activity is maintained (i.e., catalyst is in natural environment).
4) Analytical multistep enzyme sequences are already present.

Table II. Summary of heterogeneous biocatalysts used and types of analyses

Biocatalyst	Analytes
Intact organelles, membranes Intact whole cells (bacterial, tissue)	Amino acids Hormones Nutrients Antimicrobial agents Environmental toxins Inorganic ions

It should be noted that attempts to extract the deiodinating activity from the microsomal fraction have been unsuccessful and, therefore, the only approach to using this bioactivity is to use the intact microsomal system.

The application of immobilized microbial cells on electrode surfaces has given rise to "bacterial electrodes" sensitive to a variety of substances. These electrodes are characterized by prolonged lifetimes over previous isolated enzyme based electrodes for these same substrates. A glutamine sensor has been prepared by immobilizing the bacterial strain *Sarcina flava* on the surface of an ammonia gas sensing electrode (3). The electrode exhibits complete selectivity for L-glutamine over all other amino acids and has a useful lifetime of over 3 weeks. This is considerably longer than the 1-day reported for the isolated bacterial glutaminase used to prepare a glutamine enzyme electrode (9). Similarly, other bacterial electrodes have been prepared for aspartate (4) and arginine (5). A nitrate selective bacterial electrode has been developed (6), utilizing the bacterium *Azotobacter*, which reduces nitrate to ammonia at the surface of an ammonia gas sensor. The electrode functions reproducibly over a wide concentration range and can be employed for nitrate determinations in environmental water samples, without interferences from other anions.

An alternate means of preparing bioselective sensors may lie in the use of intact tissue cells. A porcine kidney slice has been used in conjunction with an ammonia gas probe to develop another glutamine selective electrode (8). Figure 2 illustrates the typical response and selectivity of this biosensor. Once again, this electrode exhibits complete selectivity for glutamine over all other amino acids and has a lifetime of over one month. Sodium azide is used to preserve the kidney cells from microbiological attack. This sensor has been used to directly determine glutamine in complex biological samples (10).

It is evident that gas-sensing electrodes are quite popular as base probes in the development of these and other biosensors. The ammonia electrodes used in this work have been commercial models, which incorporate pH glass membranes as inner sensing elements. In an effort to reduce the size, cost, detection limits and fragile nature of current ammonia detectors, a new type of miniature ammonia gas sensor has been developed (11). Figure 3 schematically illustrates this new gas probe. The electrode employs a plastic ammonium ion sensitive membrane as the inner sensing element and will be used to prepare more simplified biosensors. This ammonia electrode can also be used for direct ammonia determinations in environmental water samples.

In the future, electrode based heterogeneous biocatalytic assays will certainly be extended to determine other important amino acids, hormones, nutrients, etc. by using appropriate cellular substances. In addition, an even more exciting possibility will be the measurement of antimicrobial substances by determining their inhibitory effect on bacterial metabolic rates. With the proper choice of biological systems, it should be possible to develop simple and reliable electrode based bioassays for heavy metals, antibiotics, and other toxic substances.

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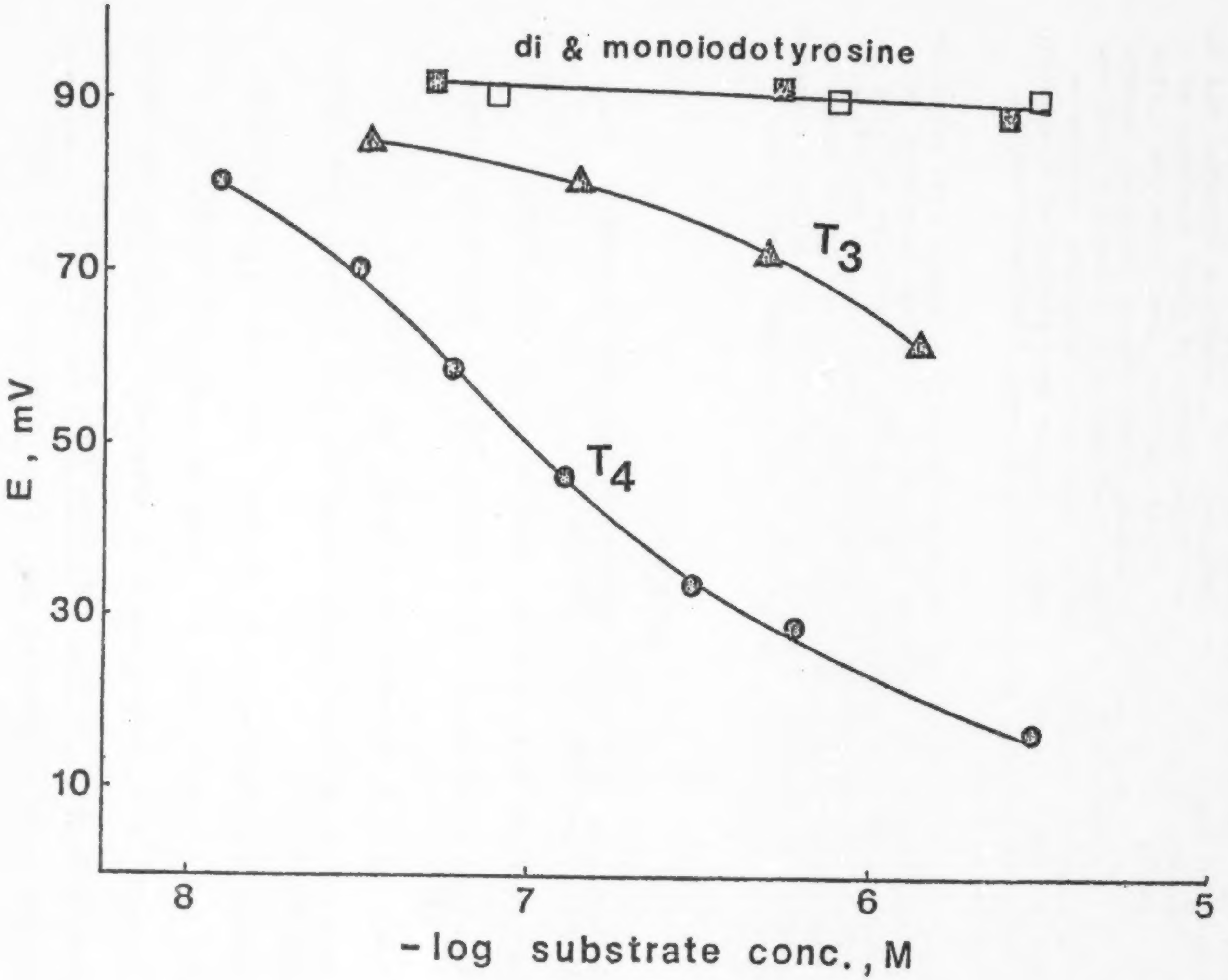

Figure 1. Response and Selectivity of Iodide Electrode Microsomal Thyroxine Measurements.

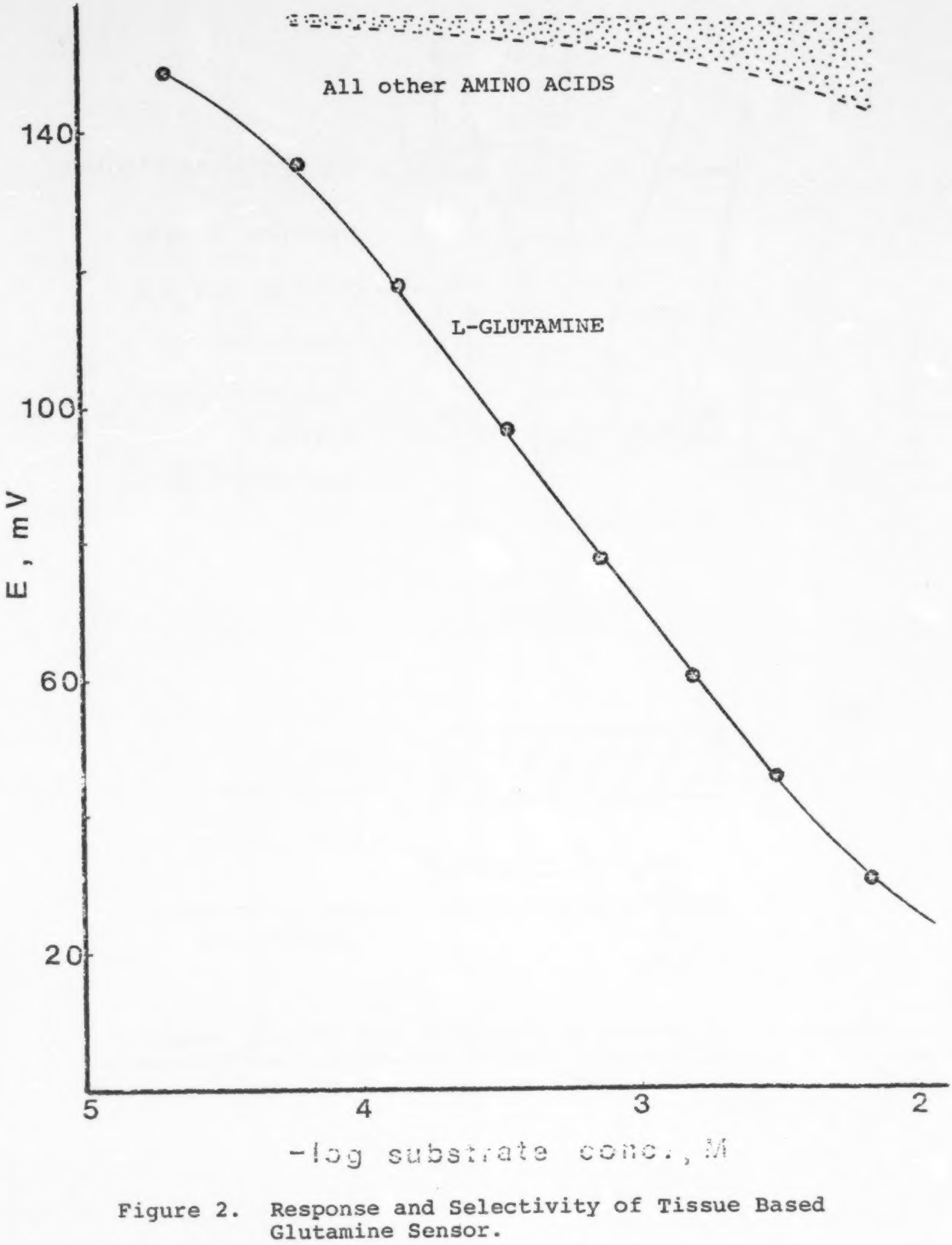

Figure 2. Response and Selectivity of Tissue Based Glutamine Sensor.

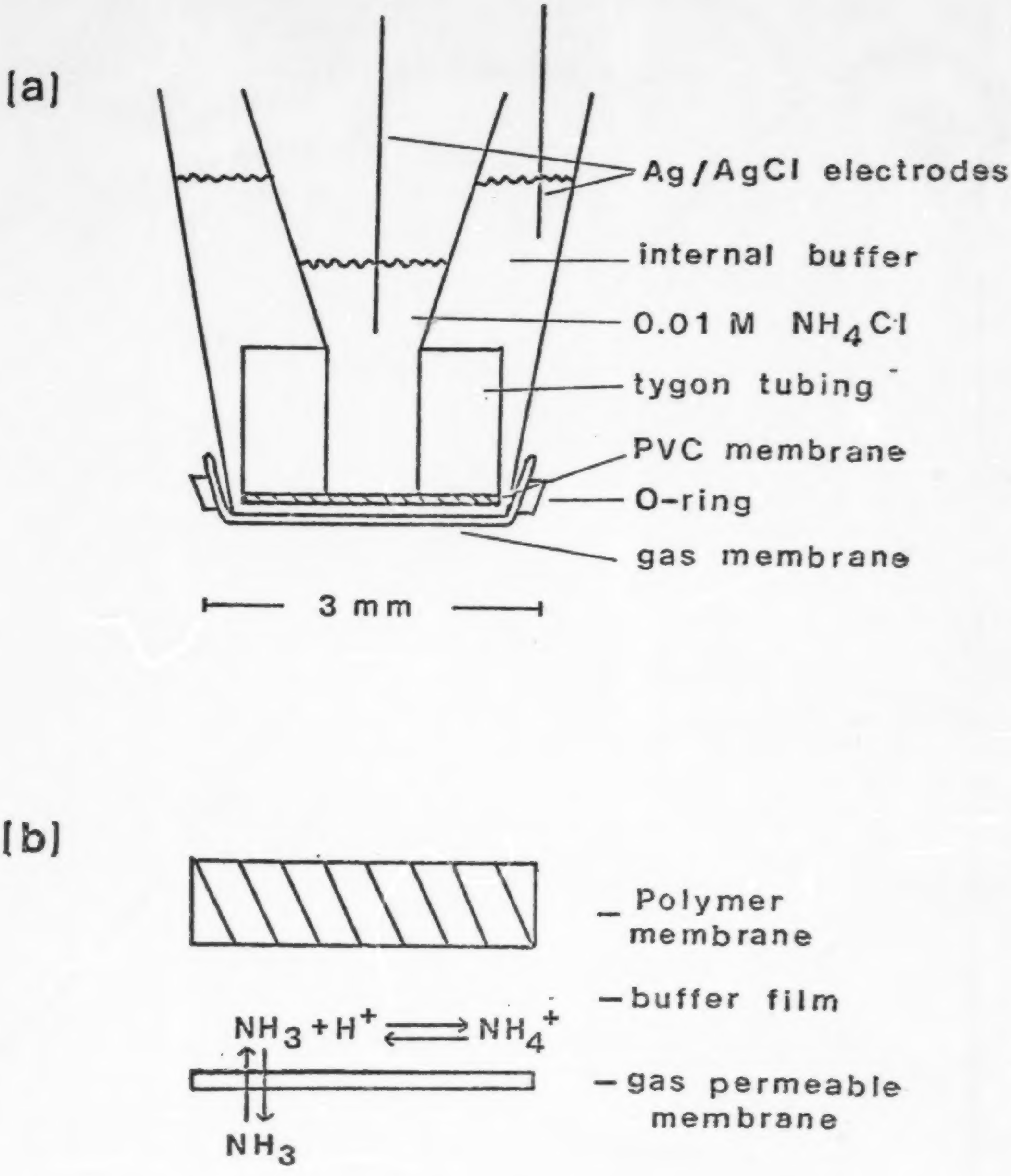
[a]
Ag/AgCl electrodes
internal buffer
0.01 M NH_4Cl
tygon tubing
PVC membrane
O-ring
gas membrane
3 mm
[b]
Polymer membrane
buffer film
$NH_3 + H^+ \rightleftharpoons NH_4^+$
gas permeable membrane
NH_3

Figure 3 a) Schematic diagram of new ammonia sensor.
b) Expanded view of sensing tip with response processes.

UTILITY OF THE MICROTOX LUMINESCENT BACTERIAL ASSAY FOR THE RAPID ASSESSMENT OF AQUATIC POLLUTION

BY

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INTRODUCTION

In 1979, the author described a new method for the quick assessment of toxicity in aquatic samples.[1] This bioassay, which utilized luminescent bacteria as the bioassay organism, displayed potential as a rapid, reliable, and inexpensive method for assessing the toxicity of water samples. The results outlined in this communication compare the luminescent bacterial assay (designated as Microtox) with conventional bioassays.* In addition, a more rapid and precise method for conducting the bacterial luminescent assay is described. The instrument is illustrated in figure 1.

Description of Microtox Toxicity Analyzer--The Toxicity Analyzer is equipped with a rotary shutter built around a photomultiplier tube adjacent to a temperature controlled reaction chamber. This reaction chamber is provided with adjustable temperature control over the range of 10°C to 25°C. The instrument is also equipped with a fifteen-well incubator chamber which was controlled within ± 0.3°C of the selected reaction chamber temperature. The reaction chamber was controlled at 15°C in all assays reported here.

The photometer was equipped with an air pump and a special drying chamber. Air was pumped through a drying chamber for distribution to incubator and reaction chambers, preventing fogging of cuvettes and minimizing water condensation in the incubator wells. The photometer was connected to a 10-inch chart recorder and all values of light levels were obtained from the chart recordings.

Reagents used for the assay--The bioluminescence assay required the use of 3 specially prepared reagents.

1. Freeze-dried luminescent bacteria -- This preparation consisted of freeze dried *Photobacterium phosphoreum* NRRL B-11177 (previously referred to as *P. fischeri* (ref. 1). When reconstituted with one ml water, the viable bacterial population is approximately 10^8 per ml. This reagent was used for only 1 hour, after reconstitution or for two complete assays.
2. Reconstitution solution -- This solution, used to hydrate the freeze-dried bacteria, was specially prepared distilled water, free of organic compounds.

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*Microtox is a trademark of Beckman Instruments, Inc., Fullerton, California

3. Diluent — This solution was used to dilute the reconstituted bacteria by adding 10 1 of bacterial suspension to 0.5 ml of diluent. The solution contained 2% NaCl which is necessary to osmotically protect the luminescent bacteria.

Assay Procedure—All samples to be tested were adjusted to 2% ± 0.2% NaCl by the addition of analytical grade NaCl. The samples were diluted using diluent solution such that the following concentrations were tested: 50, 32, 18, 10, and 5%. These diluted samples plus a clean water control (diluent only) were precooled to 15°C in the incubator wells. The Microtox reagent was hydrated with 1 ml of reconstitution solution precooled to 3°C, and the bacteria transferred to testing cuvettes containing 0.5 ml of diluent equilibrated to 15°C. Initial light measurements were made for each cell suspension. Aliquots (0.5 ml) of sample dilutions and the control were then added to the appropriate cell suspension cuvettes. After 5 minutes, final light measurements were made for each cuvette. The control was used to correct the sample for the time dependent drift in light output.

Calculation of EC50—Microtox data reduction was more precise when the gamma function (2) was used in place of percent light decrease which was used previously (1). Gamma ( ) is the ratio of the amount of light lost to the amount of light remaining (figure 2). Thus, for the case in which a 50% light reduction is observed (EC50), = 1. The procedure for calculating EC50 is as follows:

A blank ratio (BR) was determined by measuring the light intensity of the control cuvette prior to addition of diluent (B_o) and five minutes after diluent addition (B_5). This ratio was applied to initial light intensity readings (I_o) of the cell suspensions prior to sample addition. BR corrects I_o values for drift and other effects of diluent addition. This enabled the measurement of a true base line and thus the isolation of toxicity effects alone from the sample.

$$B\,R = \frac{B_5}{B_o}$$

$${}_cI_o = I_oBR$$

The corrected initial light output (${}_cI_o$) and 5-minute light output values (I_5) were used to calculate Γ_5 for each sample dilution.

$$\Gamma_5 = \frac{\text{corrected light lost}}{\text{light remaining}} = \frac{{}_cI_o - I_5}{I_5} = \frac{{}_cI_o}{I_5} - 1$$

Values for Γ_5 were plotted as a function of sample concentration on log-log graph paper. EC50 was determined by the intersection of a best fit line with $\Gamma_5 = 1.0$.

Procedure for Determination of Reproducibility—The reproducibility of this assay procedure was assessed by testing concentrations of sodium lauryl sulfate (SLS) (0.3, 1, 3 and 10 mg/liter). Tests were performed with three different lots of Microtox reagent by three technicians on different instruments. Each technician performed nine assays with each reagent lot for a total of 81 determinations of EC50.

Assay of Pure Compounds--Compounds were dissolved in the 2% NaCl diluent and appropriate dilutions prepared for testing. Those compounds which had limited water solubility (e.g., Aroclor 1242) were mixed with diluent for 2 days in a separatory funnel and the water phase removed for testing. It was assumed that this procedure saturated solutions. Literature values for concentrations of saturated solutions were used for the results reported.

Assay of Complex Effluents--Complex effluents tested were obtained from various industrial and municipal sites. These samples were assayed using conventional fish and invertebrate assay procedures while concurrently assayed using the Microtox procedure. These comparative assays were performed by government, academic, and industrial laboratories. These laboratories have been evaluating the luminescent bacterial assay and have fish-testing capabilities.

RESULTS

Variation of Microtox Assay--All 81 determinations of EC50 were used to calculate a mean value of 1.57 mg/Liter. The data displayed a variance of 0.08 and a standard deviation of 0.28 mg/Liter. The coefficient of variation for all EC50s was 18.2%. Coefficient of variation within lots were between 6 and 10%. There were no significant differences between operators and instruments.

Assay of Pure Compounds--Pure compounds which were assayed with Microtox are listed in table 1. Fish LC50 data were not experimentally determined but were obtained from the literature (4-11). In most cases a range was reported since the data were determined using several species and/or different testing periods. These data are presented graphically in figure 3. If the Microtox and fish data displayed equal sensitivities to the compound tested, all the data points would fall on the line (slope=unity) in the figure. Compounds which fell below the line were more sensitively detected by the fish bioassay, while those compounds which fell above the line were more sensitively detected by the Microtox method.

Complex Effluents--Data in table 2 present the side-by-side comparisons of the two methods, used to test effluents from industrial and municipal plants. Samples were coded because of the sensitive nature of the information. EC50 data reported as greater than 50% were estimated by extrapolation. These complex effluent data were plotted in figure 4 such that correlation could be estimated in the same manner as for pure compounds data. Table 2 contains more EC50 data than were plotted in figure 4, but those samples which gave no quantitative effect with both assay methods could not be presented graphically.

DISCUSSION

When comparing Microtox and fish sensitivities (figure 3), the trend was one of good general agreement between these two methods of bioassay. Defined toxicants were selected for an initial comparison of sensitivities since pure compounds could be assayed quickly with Microtox and fish data were readily available from the literature. The data suggest that 5-minute Microtox EC50 data are comparable with fish 24- to 96-hour data. These data also suggested the need for a direct experimental comparison using identical samples, particularly complex effluents.

The data presented in table 2 and figure 4 show good agreement between EC50s for luminescent bacteria and LC50s for fish. It is important to note, however, that there was no rigorous attempt to account for differences in test conditions such as temperature, pH, water hardness, and other factors which might affect results. Some samples had coloration which may have caused nonspecific light decrease by light absorption. This is particularly true of reds and browns which absorb strongly in the 480-nm region, which is the peak of the bioluminescence. Sample number AA20 was dark brown in color and is the only sample suspected of a significant color interference. Samples AW24 (dark blue) and AA33 (light brown) showed no apparent effect due to color.

Close inspection of the data in table 2 shows that 19 of the samples caused no effect or were slightly stimulatory with Microtox and caused no lethality with the fish bioassay. If these data could be plotted in figure 4, it would further support good correlation between 5-minute EC50 data and fish lethality data. The data in table 2 also suggested that invertebrate data for complex effluents did not correlate well with either fish or bacterial assay results.

The modified assay procedure used to generate data in this report has shown several advantages. The procedure incorporates the simultaneous assay of a control as well as dilutions of sample. This permitted challenge of bioassay organisms, at the same time following reconstitution, and thus assured that the bacteria were physiologically identical when challenged with control-plus-sample dilutions. This improved assay procedure considerably shortened assay time. The entire assay including dilutions of the sample could be accomplished in 30 minutes from the time samples were collected.

The reproducibility experiments using SLS represent a worst case since the test included different operators, instruments, and production lots of reagent. In addition, SLS represents a class of toxicant in which reaction rate is still rapid at 5 minutes. This fact made end-point precision difficult and thus entered into the reproducibility of the assay. It is felt, however, that a coefficient of variation of 18.2 percent is acceptable variability for a biological assay.

CONCLUSIONS

The Microtox bacterial luminescence bioassay method was evaluated, and the data procedure found to be a reliable method for determining acute toxicity for complex effluents. The 5-minute bioassay procedure was shown to agree favorably with 24- to 96-hour fish bioassays. An improved testing procedure was reported which allowed a complete determination of EC50s in 30 minutes. The Microtox system appears to be a convenient, reliable approach for rapidly determining acute toxicity of water samples.

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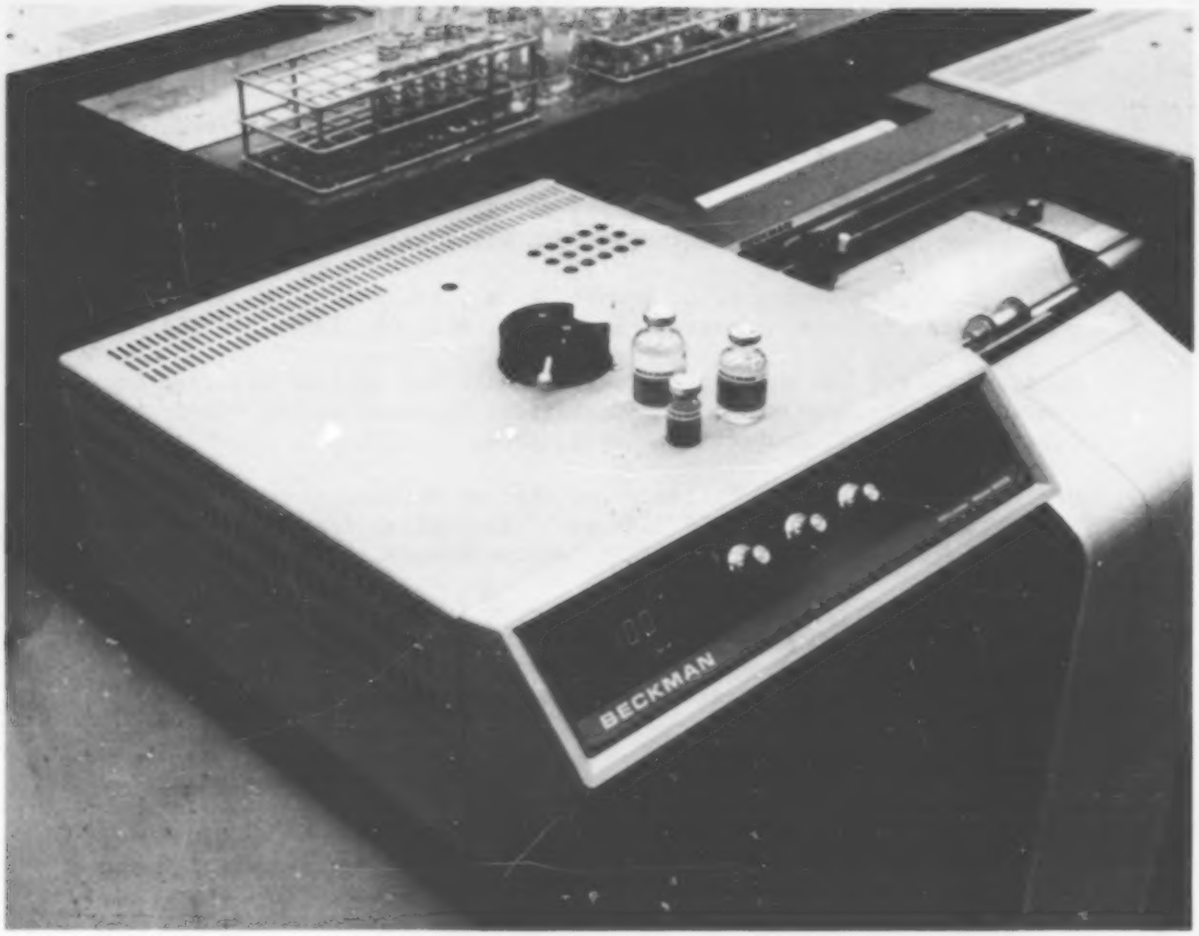
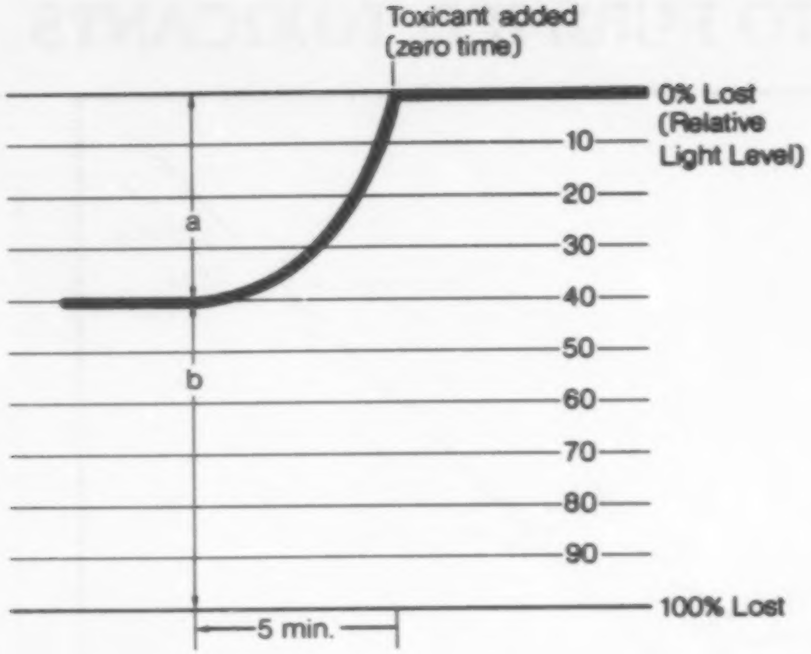


Figure 1. - Microtox Toxicity Analyzer



$$\%\Delta = \frac{\text{Light Lost}}{\text{Total Light}} \times 100 = \frac{a}{a+b} \times 100 = \frac{40}{100} \times 100 = 40\%$$

$$\Gamma = \frac{\text{Light Lost}}{\text{Light Remaining}} = \frac{a}{b} = \frac{40}{60} = 0.667$$

$$\%\Delta = \frac{\Gamma}{1+\Gamma} \times 100; \quad \Gamma = \frac{\%\Delta}{100 - \%\Delta}$$

Note: A 50% effect is equal to a gamma of 1.0.

%Δ − Γ Conversion

%Δ	Γ
0%	0
1	0.01
5	.053
10	.111
15	.176
20	.250
25	.333
30	.429
35	.538
40	.667
45	.818
50	1.0
55	1.22
60	1.5
65	1.86
70	2.33
75	3.0
80	4.0
85	5.67
90	9.0
95	19
99	99
100%	∞
%Δ	Γ

%Δ = % Decrease of Light Output

Figure 2. - Comparison of Gamma vs. % Decrease

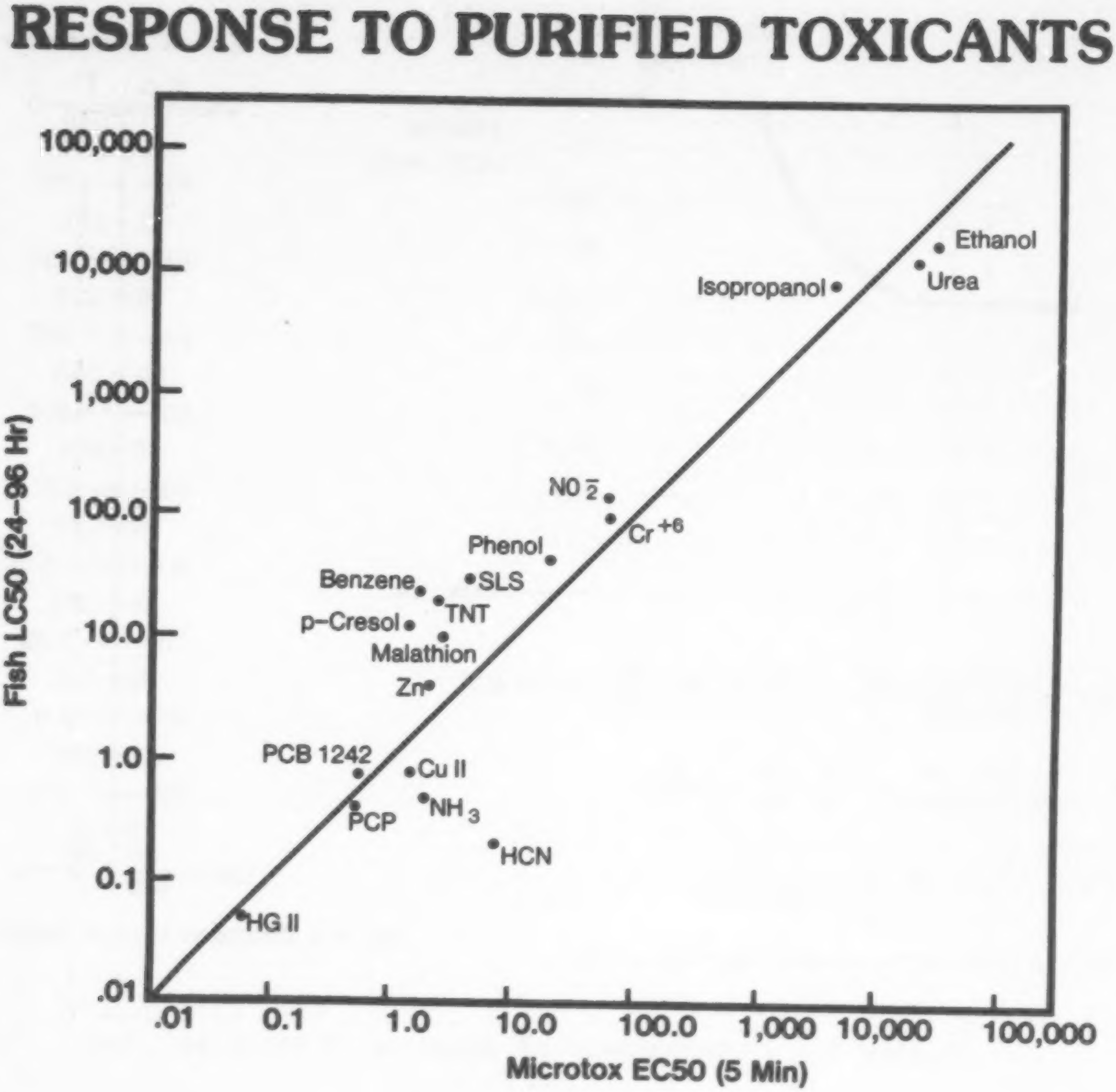


Figure 3. Comparison of Microtox pure compound EC50 data with fish LC50 data obtained from the literature.

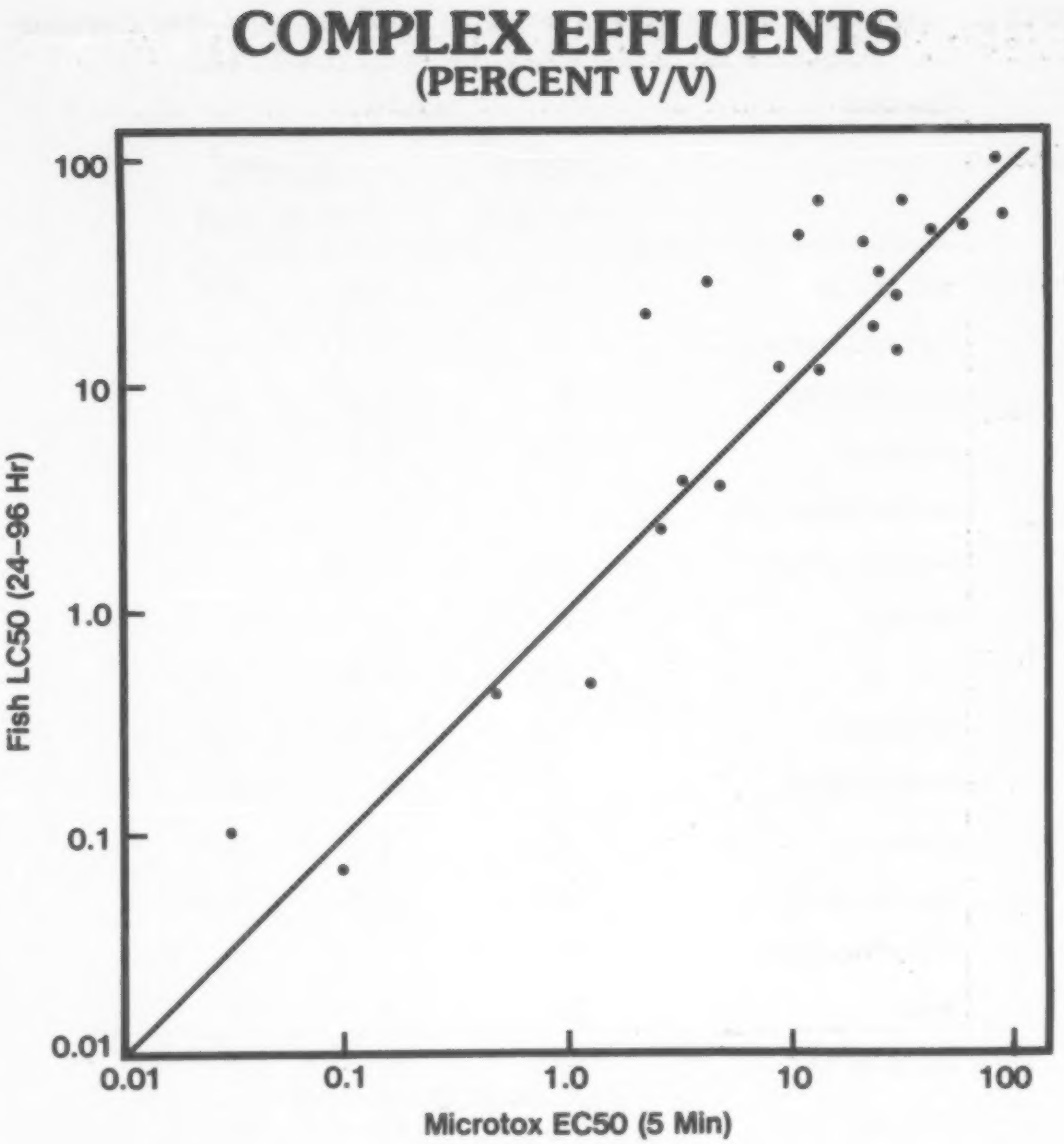


Figure 4. - Comparison of Microtox EC50 data with fish LC50 data using complex effluent samples.

TABLE 1 - COMPARISON OF MICROTOX EC50 AND FISH LC50 DATA USING PURE COMPOUNDS

Toxicant	mg/Liter	
	Microtox™	Fish Assay[a]
	5 min. EC50	24-96 hr. LC50
Mercury II	0.065	0.01 - 0.9
Pentachlorophenate	0.5	0.21 - 0.6
Aroclor 1242	0.7	0.3 - 1.0
p-Cresol	1.5	3.5 - 19
Sodium Lauryl SO_4	1.6	5 - 46
Ammonia (free)	2.0	0.068 - 8.2
Benzene	2.0	17 - 32
Zinc II	2.5	0.24 - 7.2
Malathion	3.0	0.07 - 19.5
Formaldehyde	3.0	18 - 185
Copper II	8.0	0.1 - 10.7
Cyanide (HCN)	8.5	0.1 - 0.44
Trinitrotoluene	20	26
Phenol	25	9 - 66
Chromium VI	70	29 - 133
Nitrite	420	19 - 230
1-Butanol	3300	1940
Isopropanol	42,000	4200 -11,130
Urea	24,000	12,000
Ethanol	31,000	13,500

[a] values obtained from references 4-12

TABLE 2 - Same Sample Comparison of Complex Effluents[a]

SAMPLE CODE	LUMINESCENT BACTERIA[b]	FISH		INVERTEBRATES	
	EC50[c]	LC50[c]	TYPE[d]	LC50[c]	TYPE[d]
WP26	0.032	0.1	F96RT	--	--
WP27	0.2	0.07	F96RT	--	--
WP28	0.48	0.46	F96RT	--	--
WP25	1.3	0.5	F96RT	--	--
AA20	1.6	21.7	S24FM	100	S24D
KP40	2.6	2.4	S96FM	--	--
AM 7.3	3.2	3.5	S24FM	--	--
KP42	3.5	3.5	S96FM	--	--
KP43	4.3	29	S96FM	--	--
AW21.1	7.0	19	S24FM	<5.6	S24D
AP11.1	11.5	48	S24FM	42	S24D
KP41	13	12.2	S96FM	--	--
AP11.2	15	68	S24FM	Partial[f]	S24D
AC 6	21	17.9	S72FM	>18	S72D
AW22	21.8	42	F96FM	--	--
AC34	24	13.5	S24SM	21.5	S24MS
AC 5	25	32	S24FM	<56	S24D
AP16.3	40	66	S24FM	--	--
AC23	48.5	45	S96SM	35	F96MS
AF36	60	NL[e]	S24FM	--	--
AP16.1	62	52	S24FM	--	--
AC 3	62	NL	S72FM	39.7	S72D
AC 1	68	NL	S72FM	61.4	S72D
AM 7.4	70	100	S24FM	--	--
AM 7.2	74	NL	S24FM	NL	S24D
AC43	82	66	S24FM	80	S24D
AC 8	>100	21.3	S24FM	27.2	S24D
AC44.2	>100	74	S24FM	--	--
AM 2	>100	NL	S72FM	35	S72D
AC12.2	>100	NL	S24FM	Partial	S24D
AC12.1	>100	NL	S24FM	--	--
AW21.2	NE[g]	51	S24FM	100	S24D
AM 7.3	NE	56	S24FM	38	S24D
AC 4	NE	65	S24FM	78	S24D
AP16.2	NE	75	S24FM	Partial	S24D
AC45	NE	91	F96FM	23	F96D
AC37	NE	NL	S24FM	42	S24D
AM 7.5	NE	NL	S24FM	80	S24D
AM10	NE	NL	S24FM	100	S24D
AW14	NE	NL	S24FM	Partial	S24D
AC29	NE	NL	F96SM	NL	S96MS
AC30	NE	NL	F96SM	>56	F96MS
AH18	NE	NL	S24FM	NL	S24D
AW24	NE	NL	F96FM	NL	S48D
AF35	NE	NL	F96B	--	--
AA33	NE	NL	F96SM	NL	S96MS
AC31	NE	NL	F96SM	--	--
AC39	NE	NL	S96B	--	--
AB32	NE	NL	F96SM	NL	F96MS
AC44.1	NE	NL	S24B	--	--
AC19	SS	NL	S24FM	81	S240

TABLE 2 - Continued

SAMPLE CODE	LUMINESCENT BACTERIA[b]	FISH		INVERTEBRATES	
	EC50[c]	LC50[c]	TYPE[d]	LC50[c]	TYPE[d]
AP16.4	SS	NL	S24FM	Partial	S24D
AX13	SS	NL	S24FM	Partial	S24D
AW15	SS	NL	S24FM	Partial	S24D
AH 9	SS	NL	S24FM	Partial	S24D

[a] Data furnished by independent evaluators(listed in acknowledgments.)

[b] 5 minute data

[c] Percent Effluent causing 50% effect or 50% lethality.

[d] Assay type: F=Flow thru; S=static; #=hours; RT=rainbow trout; FM=fathead minnow; B=bluegill; SM=sheepshead minnow; D=daphnia; MS=mysid shrimp

[e] NL = no lethality

[f] Partial = insufficient deaths for calculation of LC50

[g] NE = no significant effect

[h] SS = slight stimulation

THE USE OF ALGAE AS BIOLOGICAL ACCUMULATORS FOR MONITORING AQUATIC POLLUTANTS

by

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INTRODUCTION

Increasing awareness of and concern about the degradation of water has resulted in a growing body of knowledge concerning the aquatic environment in the natural state and stressed by pollution. The types, concentrations, and persistence of aquatic pollutants have been major research interests in the past decade. Kaiser (1971) estimated that as analytical detection limits increased from the ppm to the ppb range, the identification of trace chemical components could increase from 10^3 to 10^6. While the accuracy of Kaiser's prediction cannot be determined, over 200 organics have been identified in drinking water alone. Sheldon and Hites (1979) identified over 100 organics present in the Delaware River with a significant number of the compounds originating from anthropogenic sources. A similar situation has been found for many other rivers in industrialized areas. The development and application of computerized gas chromatographs/mass spectrometers equipped with capillary columns permits the separation and subsequent confirmation of complex mixtures of trace organic pollutants.

Concern about the effects of many heavy metals in the aquatic environment is clearly demonstrated by their inclusion in the Environmental Protection Agency Priority Pollutant List. Routine detection limits for metals have been extended through the use of the graphite furnace, atomic absorption spectroscopy, and plasma emission spectroscopy.

As a result of these improved procedures, a rapidly expanding body of literature describing levels of aquatic pollutants in both abiotic and biotic environments has developed. Current research is focusing upon the determination of the biologically active forms of pollutants at the μg/l level and evaluating synergistic and antagonistic interactions among pollutants within organisms. The levels at which pollutants are available to biota are not necessarily levels that can be detected analytically or the levels that theoretically would bioaccumulate. A laboratory estimate of potential accumulation of aqueous organic pollutants is provided by the octanol-water partition coefficient (Chiou et al., 1977). In the environment, however, there are many competing mechanisms or environmental influences that could either increase or decrease pollutant availability (Hassett and Anderson, 1979). The levels, rates and persistence ultimately determine this availability. Biologically available levels of metals depend upon metal-complexation with inorganic and organic substances which may be present, precipitation or solid sorption (fig. 1).

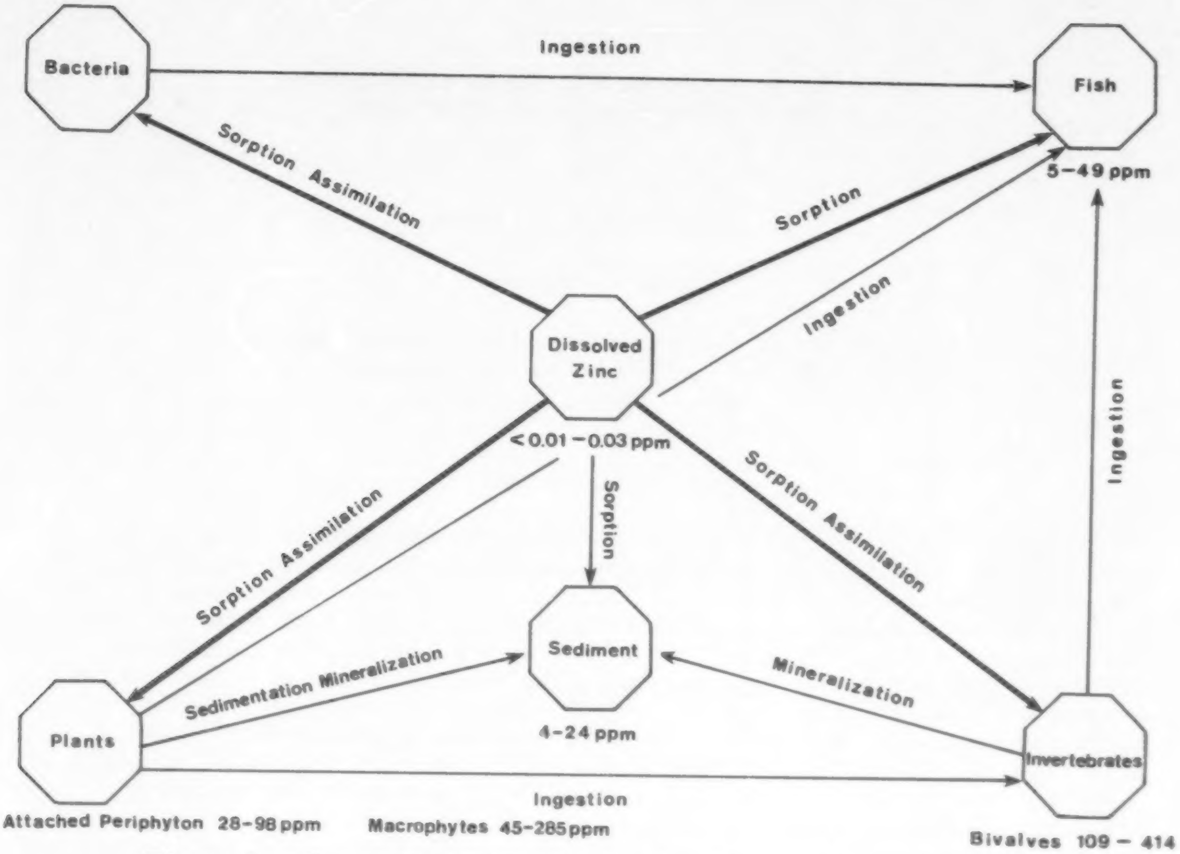
Bacteria
Ingestion
Fish
Sorption Assimilation
5-49 ppm
Sorption
Ingestion
Dissolved Zinc
< 0.01 - 0.03 ppm
Ingestion
Sorption
Sorption Assimilation
Sorption Assimilation
Sediment
Sedimentation Mineralization
Mineralization
4-24 ppm
Plants
Invertebrates
Ingestion
Attached Periphyton 28-98 ppm
Macrophytes 45-285 ppm
Bivalves 109 - 414

Figure 1.--The partitioning of zinc in the aquatic environment.

Concern about the quality of surface waters and the origin of specific pollutants has promoted the development of remote samplers and monitors to obtain time-integrated water samples for analysis. While these units capably collect and monitor the discharge of industrial wastes, there would appear to be little application in monitoring certain aspects of industrial wastes, long-term general water quality, and especially the biological availability of pollutants.

It is reported in the literature that analyses of a wide variety of parameters are possible automatically with in-situ monitors, when in fact, the analyses of only a few parameters by in-situ sensors have been proven reliable (Pijanowski, 1974). These include conductivity, temperature, depth, dissolved oxygen, and pH. Some instruments offer specific ion sensors but, because of the lack of specificity, these sensors are useful for only limited and specialized applications. Each specific ion electrode must have a reference electrode and amplifier. Ingols and Craft (1975) point out that the choice of specific ion electrodes for monitoring river water quality will depend upon concentration range, and the ease and frequency of available service for the sensor. They felt that although specific ion electrodes are useful in the laboratory, field use is extremely limited.

Concurrent with the development of monitoring devices to measure general water quality and the presence of toxic substances has been the increased use of biomonitoring, i.e., the use of organisms to determine the state of an aquatic environment (Cairns and Dickson, 1973). Weber (1973) divided biological monitoring methods into three categories: "methods for studying the effects of pollutants on indigenous communities of aquatic organisms in receiving waters, methods employing captive organisms or controlled conditions to determine the toxicity or other biological effects of substances or conditions in effluent or receiving water and methods employing bioaccumulation or biomagnification to detect or monitor trends in the concentration of pollutants in surface waters." However, pollutant profiles obtained from biological organisms can be misleading due to the effects of extraneous variables on the physiology of the organisms used and because different organisms acquire different components of the total pollutant load, resulting in different profiles for each organism. For an organism to be useful for biomonitoring it must meet certain criteria. In a summary of these criteria Phillips (1977) stated the following.

1. The organism should be exposed to subacute levels of the pollutant.
2. The organism should have limited spatial mobility.
3. Significant abundance of the organism should exist in the study area.
4. The organism should have sufficient longevity to satisfy study objectives.
5. The organism must provide sufficient biomass for proper pollutant detection.
6. The orgnism should be adaptable to permit laboratory accumulation studies.
7. Monitoring program should be adaptable to both fresh and brackish water.
8. The organism should exhibit a pollutant concentration factor that permits detection.
9. A known relationship should exist between accumulation and exposure concentrations.
10. The organism should demonstrate a relationship between uptake and exposure concentration in the study area.

The intent of this paper is to present an overview of possible biomonitoring uses of algae as aquatic pollutant bioaccumulators. A discussion of the factors that determine bioavailability of metals to bivalves and algae as well as the general relationships between exposure concentrations of metals and algal uptake is included. Original data from studies using algae growing on artificial substrates to monitor point-source trace metals and nonpoint-source organics are presented.

BIOLOGICAL AVAILABILITY OF METALS

Estimating the biological availability of metals in water column samples by direct measurements presents difficulties because of many competiting reactions, including complexation, precipitation, and sorption. Also, frequent sampling is required to establish any water column trends.

Luoma and Jenne (1976) attempted to isolate by chemical extraction procedures that fraction of cadmium, cobalt, silver, and zinc in sediments biologically available to deposit-feeding clams. They concluded that the type of extractant solution needed to estimate biologically available metals varied depending upon the metal and its form in the sediments. It is unlikely that any single analytical procedure will describe that fraction of metals in sediments that is biologically available.

In contrast to water or sediment analysis, a direct indication of the biological availability of metals can be derived from an analysis of biological organisms, the single most important advantage of such an analysis, according to Phillips (1977). He extensively reviewed the use of biological organisms to monitor aquatic metal pollutants and pointed out that indicator organisms have been used by many researchers to monitor time-averaged concentrations of trace metals (Phillips, 1977).

Pollution profiles obtained from the analysis of organisms will vary depending upon habitat and their interaction with the physico-chemical environment. Use of organisms without knowledge of mechanisms of interaction with the environment could result in an improper interpretation of the study results with respect to pollutant loading (Phillips, 1979).

Metal accumulation by oysters has been reported to be not necessarily a function of a particular source but also a function of location of the organisms in the estuary (Huggett et al., 1976). This may be due in part to the nonhomogeneous nature of sediments, which may or may not reflect metal activity in the water column depending upon water column physico-chemical removal mechanisms to the sediments. Batley and Gardner (1978) point out that in the mixing zones of estuaries the most significant changes in metal speciation will occur.

Greig and Wenzloff (1978) studied the uptake of cadmium, copper, silver, and zinc by the oyster Crassostrea virginica and found that only cadmium and silver were obtained directly from the water column. Copper was suggested to have been derived directly from seawater and/or food, suspended in sediment, or from sediments directly. No relationship was found to exist between zinc concentrations and the environment. Greig (1979) showed that in ocean quahogs, surf clams, and oysters at least part of the total body burden of cadmium, copper, and silver is directly available from the water column. What fraction this represents of all environmental inputs was not reported. Phillips (1979) investigated the accumulation of cadmium, iron, lead and zinc by the mussel Mytilus edulis (L) in a field study. No relationship was found to exist between tissue metal concentrations and water concentrations.

Anderson (1977) studied the concentrations of cadmium, copper, lead, and zinc in six species of freshwater clams found in the Fox River. Considerable variation in metal concentrations was found to exist between taxa and between individuals of the same taxon. A correlation was found between organism metal concentration and sediment metal concentrations. It was suggested that since the gills act as both a filtering mechanism for food and as a respiratory organ, metal could be sorbed from both water and substrate.

It would appear from these and other studies that metals accumulated by bivalves represent an integration of metals from food, sediment, and the water column. The factoring of any particular mechanism of accumulation would apparently be very difficult because of the many interactions. Algae, on the other hand, present a different pollution profile of their environment.

Metal accumulation by algae is apparently directly related to water-column metal concentrations and, for copper at least, this relationship exists due to free ion activity. Sunda and Guillard (1976) reported that bioavailability of copper to algae is a function of free ion activity rather than total metal present and have suggested that this relationship is probably not limited to copper. A similar copper-availability relationship was also reported by Gachter et al. (1973). The free ion relationship to availability is apparently not limited to algal uptake (Sunda et al., 1978) but constitutes a fraction of the metal accumulated by other organisms. Metal activity is regulated by pH, ionic strength, particulate carbon, and chelating agents. As discussed by Cross and Sunda (1978), the energy of a chemical reaction is supplied by metal free-energy or metal activity. Biological availability is also dependent upon the molecular interaction between the metal and the organism, occurring at the contact site of the organism. Conway and Williams (1979) studied the uptake of cadmium by diatoms and found significant amounts of cadmium, at least initially, were present at the organic coating of the cell wall. It was suggested that ion exchange with a constituent of the polysaccharide coating could occur. This would suggest that metal competition at the reaction site is also a key factor in ultimately determining metal uptake. Kinkade and Erdman (1975) found that the rate and level of uptake for cadmium in a variety of freshwater organisms was affected when the water was varied from soft to hard. Similar results were reported by Guthrie and Cherry (1977) where different concentrations of heavy metals altered the bioaccumulation of all metals in a natural system. These investigations suggest that metal biological-availability is regulated by interactions that can decrease metal activity.

The interaction of organic and inorganic complexes in the aquatic environment which reduces free-ion activity is difficult to measure and predict since these values will vary seasonally and from river to river and river to estuary. McCrady and Chapman (1975) found that copper added to natural river water always resulted in copper-ion concentrations that were less than theoretically calculated concentrations.

From the preceding discussion it is apparent that not only does analysis of biological organisms for metal content provide an estimate of biologically-available metal in the form of a recent history of exposure integrated over time but also reflects varying environmental factors including pH, ligands, competition of metals, and the natural background matrix.

ALGAE AS BIOACCUMULATORS OF METALS

In the analysis of a pollutant from any matrix, i.e. water, tissue, or sediment, the method of isolating the pollutant is a key consideration since it defines the compounds that will be isolated and their recovery, together with possible coextractives, may interfere with the analysis. Each isolation method has some degree of selectivity toward a specific compound, usually related to polarity, molecular weight, chemical speciation, etc. Similarly, biological systems can be thought of as a specific isolation method that defines the types of pollutants accumulated and the degree of accumulation. It is necessary, then, to understand the capabilities of bioaccumulation so that organisms may be properly used as an analytical tool in environmental monitoring.

Freshwater and marine algae have been successfully used for water quality monitoring for years by studying organismal response to changes in the aquatic environment (Cairns and Dickson, 1973; Patrick, 1973). In addition, metal accumulation by algae has been used as a tool by researchers to monitor water-column trace metals resulting from point- and nonpoint-source pollution. This approach is possible because of the proportional relationship of metal bioaccumulation to exposure concentration (Phillips, 1979). Foster (1976) reports that this relationship should hold except for grossly polluted areas. Trollope and Evans (1976) point out that since no concentration procedures are required, metal monitoring using algae can avoid many of the difficulties associated with metal analysis in very dilute solutions. The collection and use of algae is simple and inexpensive and studies can be performed on a continuous or semi-continuous basis. The pollution profile obtained is a recent historical integration or reflection of metals in the environment. Foster (1976) points out that this integration takes place over time intervals long enough to moderate the influence of fluctuations caused by short-term variations in water column metal loading.

Recent literature has reported the use of algae as biomonitors and accumulators of metals including discussions of mechanisms of metal uptake and relationships that have been used to describe this uptake.

Trollope and Evans (1976) profiled the concentration of copper, iron, lead, nickel, and zinc in freshwater algal blooms and found that differences in algal metal content were related to environmental effects. They found a linear relationship betwen zinc content of algae and that in the surrounding water, although no clear relationship was found to exist for the other metals. Biological accumulation factors ranged from 10^2 to 10^4.

Martin and Broenkow (1975) and Windom et al. (1973) reported the use of oceanic plankton to monitor areas of pollution for cadmium and mercury, respectively. It was reported that mercury accumulation was independent of biological composition. In a similar study Burkett (1975) used freshwater algae to detect the presence of mercury.

A knowledge of the relationship between metals and chemical speciation is extremely important in assessing the utility of a pollution profile obtained from algae. Skaar et al. (1974) reported that uptake of ionic nickel by a marine diatom was directly related to media concentration. Similar results were reported for zinc in freshwater algal blooms (Trollope and Evans, 1976) and for natural communities of periphyton in a closed lotic microcosm (Cushing and Rose, 1970). The uptake of mercury by a freshwater diatom (Fujita and Hashizume, 1975) and lead for a marine diatom (Schulz-Baldes and Lewin, 1976) has been described by Freundlich adsorption isotherms; $x/m = kCe^{1/n}$, where x/m is the bioaccumulated concentration, Ce the equilibrium test concentration, and k and n are constants. The fit of the data for mercury and lead accumulation to the above equation suggests that at least a portion of a metal is accumulated by passive uptake (Filip and Lynn, 1972; Parry and Hayward, 1973; and Fujita et al., 1976). Cushing and Rose (1970) showed that zinc uptake decreased with the addition of magnesium because of increased competition for adsorption binding sites. Organically complexed cadmium (Cossa, 1976) and iron (Goldberg, 1952) have been reported to be biologically unavailable to algae. Similar results have been reported for copper (Sunda, 1976), mercury (Fujita and Hashizume, 1975) and nickel (Skaar, 1974).

The majority of the previous studies used unialgal cultures run in batch. The use of artificial substrates for periphyton-growth studies has presented an alternate approach for pollutant accumualtion studies (Patrick et al., 1975, and Johnson et al., 1978). Periphyton colonizing artificial substrates have been used extensively as indicators of water quality (Patrick, 1973; Patrick et al., 1975; and Weitzel, 1979).

A device, termed the Catherwood diatometer, permitting the continuous development of diatom communities on glass slides in natural and artificial settings (fig. 2) was developed and used by Patrick. Research employing the device in natural streams indicated that 75 to 80% of diatom species found in hand collections would be found colonizing the glass substrates over a two-week exposure period. Benthic, epiphytic and planktonic diatoms are well represented on exposed slides. Use of the substrate enabled Patrick to quantify diatom community growth with respect to biomass and community structure (Patrick, Hohn, and Wallace, 1954). Variations within the structure of the communities were related to conditions such as stress caused by organic loading or toxicity in the aquatic environment (Patrick, Hohn, and Wallace, 1954, and Patrick and Strawbridge, 1963a; 1963b). Diatometer monitoring studies have been undertaken on rivers and estuaries across the United States; many of these ongoing programs have been continuous for over ten years, a few providing diatom growth records for up to 20 years.

More recntly, Patrick (1967) reported on a system for studying natural communities of diatoms under laboratory conditions. Artificial substrates were placed in artificial stream beds located in greenhouses and exposed to natural stream water supplied by pumping systems. The test chambers allowed developing diatom communities to be subjected to controlled variables. Among others, completed experiments have involved amounts and ratios of nitrogen and phosphorus (Patrick, 1967), changes in pH and temperature (Patrick, Roberts, and Davis 1968), interrelationships between temperature and manganese concentrations (Patrick, Crum, and Coles, 1969), and variations in day length and temperature (Patrick, 1971).

Patrick, Bott and Larson (1975) reported on the effects on diatom growth of vanadium, chromium, selenium, boron, nickel, and rubidium in varying concentrations. Tests employing substrates were again conducted in greenhouses during

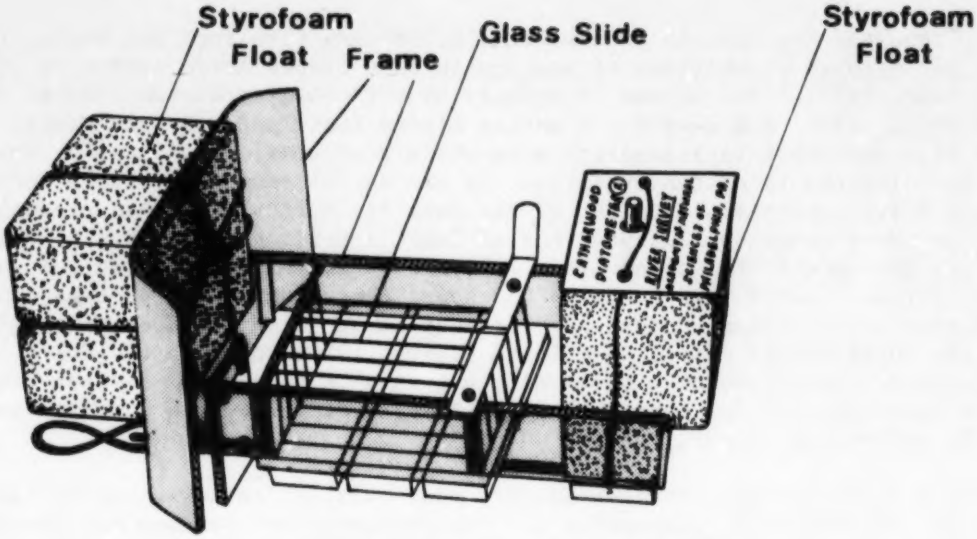

Figure 2.--Catherwood Diatometer

various seasons of the year. These experiments included analyses for accumulated test metals. Vanadium, chromium, selenite, and nickel were found to accumulate in diatoms in proportion to test water concentrations. From these studies, artificial substrates would seem particularly suited for use in biomonitoring/bioaccumulation studies, especially studies involving point-source discharge.

BIOMONITORING CHEMICAL POLLUTANTS USING ALGAE ON ARTIFICIAL SUBSTRATES

Trace Metals Study--A recent study was conducted on the Wateree River, South Carolina, to monitor trace metal levels in the area of a point-source discharge (Friant and Koerner, in press). Algal communities developing on artificial substrates were used as biomonitors in the river, which receives a secondarily treated effluent from a synthetic chemical manufacturing plant.

Catherwood diatometers were placed at three stations: a reference station 1/2 mi above the discharge (Station 1), a station immediately downstream of the discharge (Station 2), and a station 1/2 mi below the discharge (Station 3). Substrate slides were exposed in the river for two-week periods, removed and replaced for a total of 10 successive exposures from April to October 1977. Recovered slides were air dried, acid digested with nitric acid, and biologically identified. Duplicate slides were acid digested with hydrochloric acid and analyzed by atomic absorption spectroscopy for biological accumulation of antimony, chromium, copper, mercury, and zinc (Friant and Koerner, in press).

Concentrations of biologically accumulated chromium, copper, and zinc for the three study stations are shown plotted for each exposure period in figures 3, 4, and 5. The corresponding box and whisker graphs (Tukey, 1977) illustrate

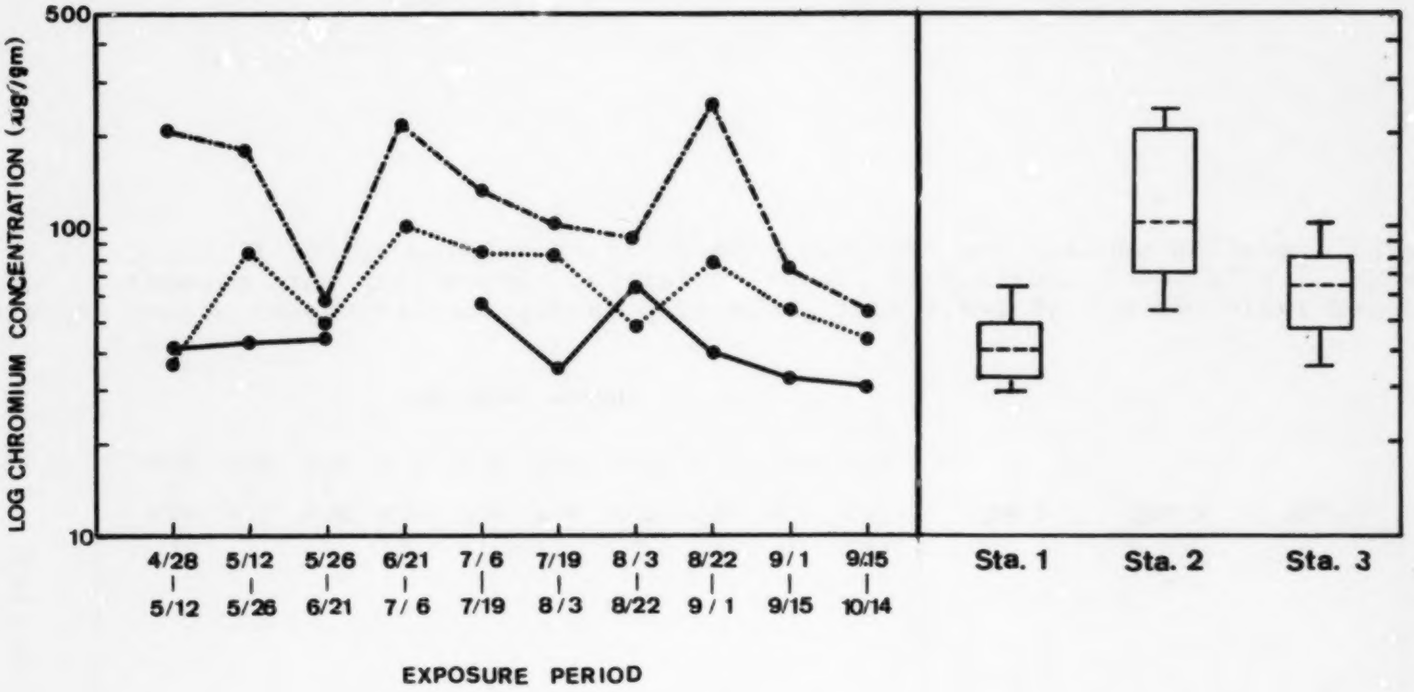

Figure 3. Chromium concentrations (µg/g) determined from algal growth and other materials removed from diatometer substrates exposed at Stations 1 (——), 2 (-··) and 3 (····) in the Wateree River, South Carolina, May through September 1977.

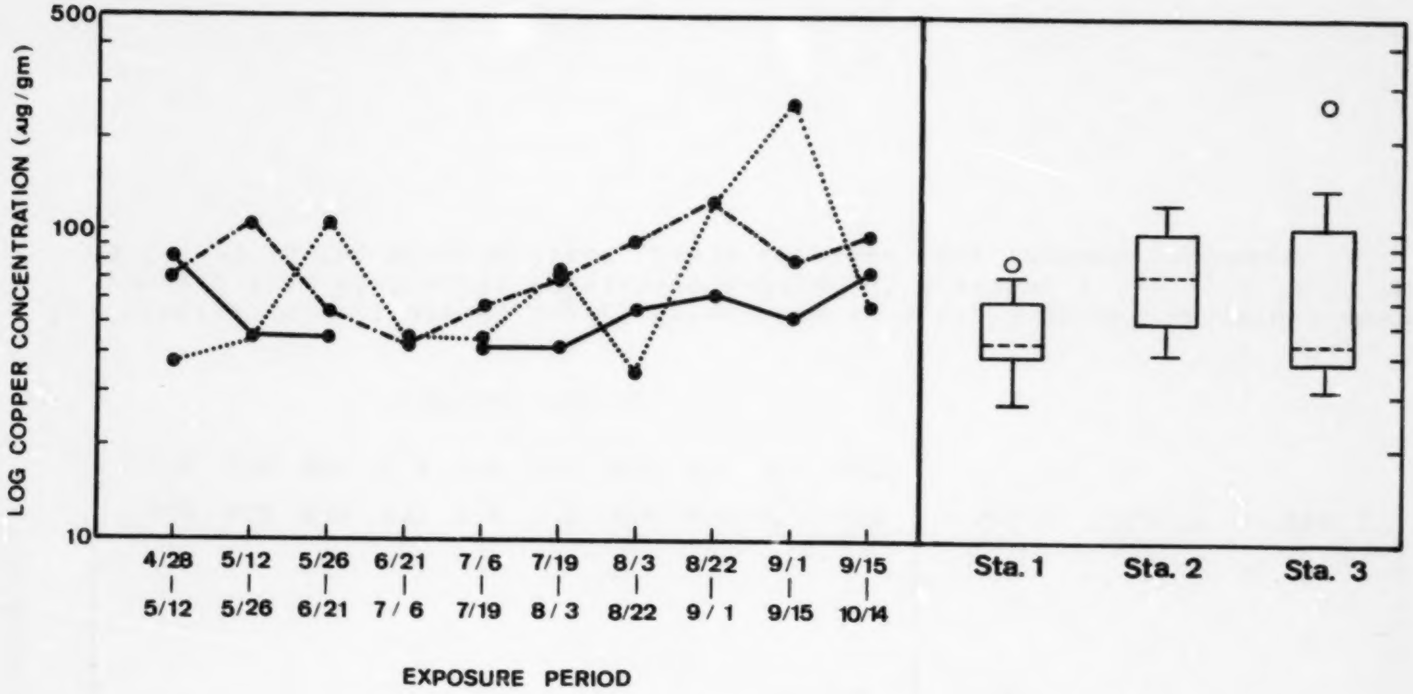

Figure 4. Copper concentrations (µg/g) determined from algal growth and other materials removed from diatometer substrates exposed at Stations 1 (——), 2 (-··-) and 3 (····) in the Wateree River, South Carolina, May through September 1977.

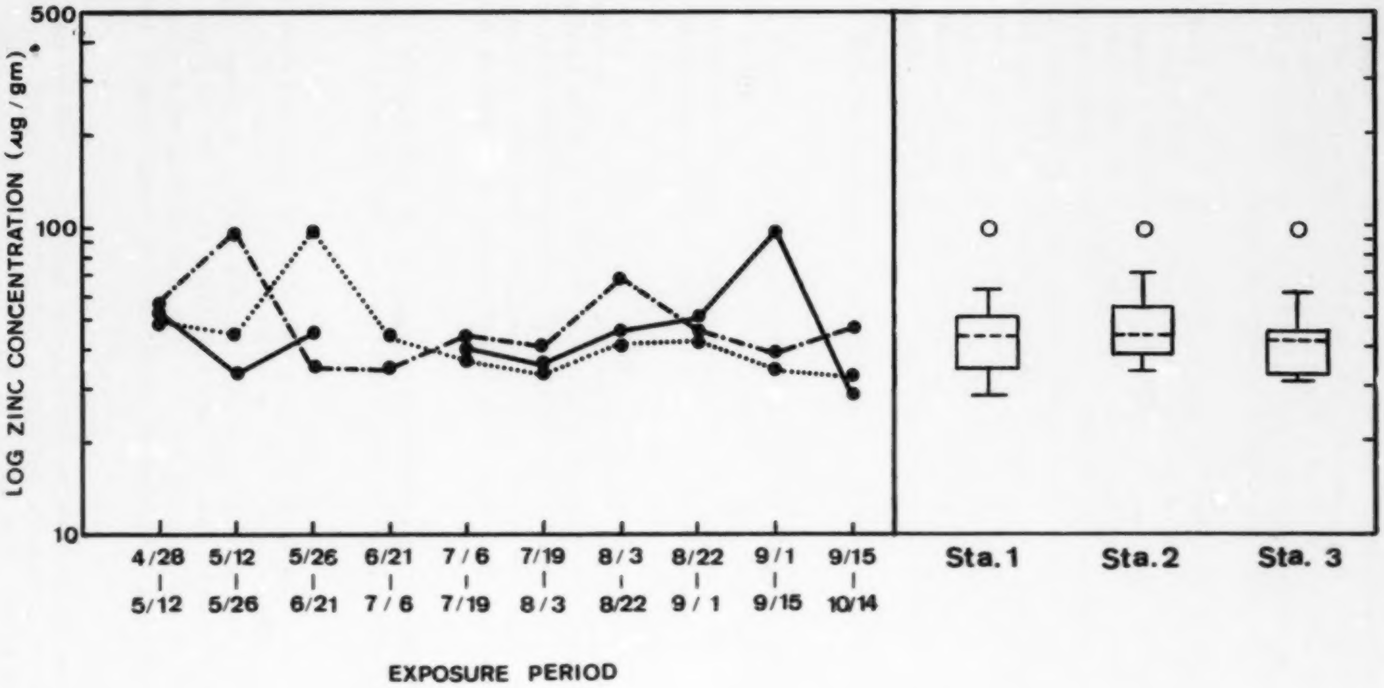

Figure 5. Zinc concentrations (µg/g) determined from algal growth and other materials removed from diatometer substrates exposed at Stations 1 (——), 2 (-··-) and 3 (·····) in the Wateree River, South Carolina, May through September 1977.

the distribution of values by range, quartiles, and median. Accumulated antimony and mercury were not found in sufficient quantity to warrant consideration in the study. To determine whether a station-related trend in metal accumulation existed, the time-series data were analyzed by a two-way analysis of variance. To account for the nonnormal distribution of values (apparent in the box and whisker graphs), a distribution-free version of the analysis of variance was used. Medians were used to express concentrations.

Statistical analysis revealed that chromium was present at Station 2 in significant amounts ($p = 0.01$) (table 1). A highly significant accumulation of chromium existed at the discharge station, Station 2, and in somewhat lower concentrations downriver at Station 3. Information concerning plant processes supplied by the chemistry plant personnel revealed that of the five metals analyzed only chromium was present in the process water and intermittently present in the treated effluent. This intermittent release of chromium may explain the large range of values for successive exposure periods determined for samples from Station 3 (fig. 3). Station 3 samples, although having higher values than the reference station samples, were similar in range. Elevated chromium levels at this downriver station were probably due to river dilution of the discharge chromium.

Analyses for other metals did not reveal any significant station-related accumulation (figs. 3 and 4).

Chemical analyses of sediment samples from the study area failed to show any statistically significant accumulation of any metal. The lack of evidence for station-related accumulations of chromium in the sediments, while finding significant station differences in bioaccumulation, suggests that the bioaccumulated concentrations reflect the water column concentrations. These data appear to present a more realistic reflection of the chromium in the discharge than routine sediment analysis would provide. The chromium concentrations determined in the diatometer analyses represented the biologically available form isolated from natural algal communities (predominantly diatoms) colonizing the substrate. The consistent station differences seen in figure 3 suggest that any single exposure period of two weeks would have detected the presence of chromium at Station 2.

Trace Organics Study--To test the feasibility of algal biomonitoring of trace organics, a study was carried out in the estuary portion of the Delaware River during the spring and summer of 1979.

A series of 30 diatometers was placed from above the city of Philadelphia at the Torresdale Water Treament Plant Intake (river mile 110) to center city (river mile 100). The diatometers were grouped and slides within a group were pooled, thus providing three discrete samples representing areas of varying river pollutant loading (Sheldon and Hites, 1979). Exposed slides were removed and replaced every two weeks during the 10-week study period comprising a total of 5 exposure periods.

Organics were extracted from samples using hexane and were profiled by gas chromatography using (1) electron capture detection for polychlorinated biphenyls and (2) flame ionization detection. The specific gas chromatographic conditions are listed in table 2. Biological sample preparation involved scraping the exposed slides followed by the sample preparation procedure shown in figure 6.

Table 1.--Medians of chromium, copper, zinc, and antimony concentrations in algae, based on 10 consecutive 2-week exposures of Catherwood Diatometers at three stations on the Wateree River, South Carolina; April through September 1977. Analyses of variance on ranks for station differentiations.

	Medians (µg/g)			Analysis of Variance		
	Sta. 1	Sta. 1	Sta. 3	Degrees of Freedom	F	p
Chromium	41	115	65	2/17	23.06	0.0001
Copper	51	77	51	2/17	2.86	0.0851
Zinc	45	45	42	2/17	0.51	0.6092
Antimony	1	1	1	2/17	0.14	0.8713

Table 2.--Gas chromatographic conditions for analysis of trace organics isolated from algae grown on artificial substrates.

ECD ^{63}Ni - Detector
Column - 3% OV-101 Chromosorb 80/100 6 ft x 1/4"
Isothermal 0 190°C
Inlet - 200°C
Detector - 350°C
Carrier - Nitrogen
Make-up - Argon/10% Methane

FID - Detector
Column - Capillary 30 meter OV-101
PTGC - 50-260°C at 7°C/min
Initial hold - 3 min.
Inlet - 200°C
Detector - 225°C
Carrier - Nitrogen

Analytical Scheme

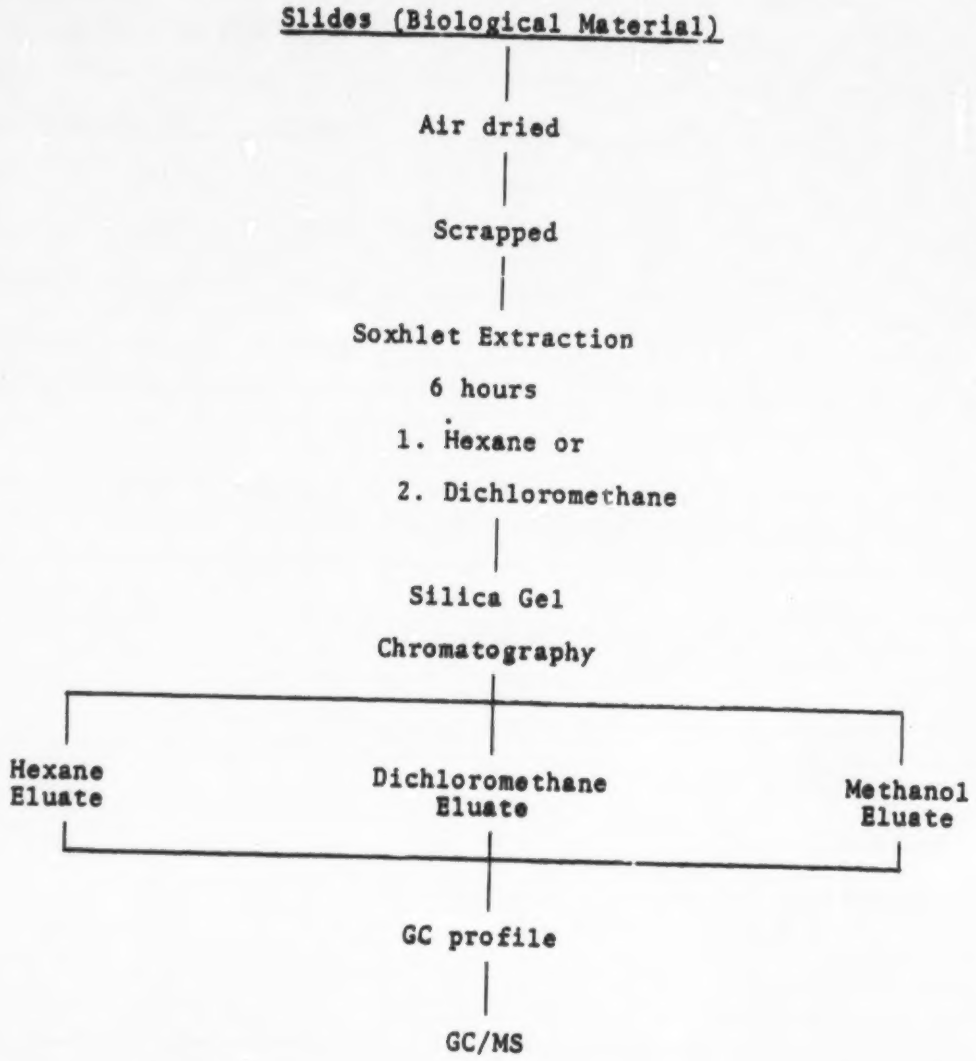
Slides (Biological Material)
Air dried
Scrapped
Soxhlet Extraction
6 hours
1. Hexane or
2. Dichloromethane
Silica Gel
Chromatography
Hexane Eluate
Dichloromethane Eluate
Methanol Eluate
GC profile
GC/MS

Figure 6. Schema for isolation and analysis of trace organics in algae grown on artificial substrates.

A typical gas chromatogram of the electron-capture pollution profile for each of the three samples, all run under identical conditions, is shown in figure 7. Curve A is from the sample representing the area above the city in the vicinity of the water treatment plant (≈ river mile 110). Curves B and C represent the areas around river mile 108 and 100, respectively, within a heavily industrialized area of the city (Sheldon and Hites, 1979). Curve D is a PCB standard, Aroclor 1254. When the standard and sample curves are compared, a high degree of similarity may be seen; there is obviously not an exact match, which suggests that the samples are mixtures of PCBs.

Looking downriver (Curves A to C, fig. 7), a station-related pollution trend is apparent. The station above the city (Curve A) was generally lowest in PCBs throughout the study and overall higher levels may be seen in the curves representing heavily industrialized areas. It aplpears that both point and nonpoint sources are contributing PCBs to the river within the city while PCB contributions upriver of the city are somewhat less. The actual concentrations of PCBs for the complete study period are currently being determined.

Flame ionization analysis for these compounds is shown on the chromatogram in figure 8. Again, a station-related trend may be seen moving downriver (Curve A to C); it is apparent that Curve B is considerably more complex than either of the other two curves. Curve B corresponds to the most heavily industrialized area along the entire stretch of the river in the study. Identification by GC-MS of some of the compounds present in this sample are shown in table 3.

Table 3.--Organic compounds present in diatometer samples from ≈river mile 108 on the Delaware River. Gas chromatogram for these samples shown as Curve B, figure 8.

Compounds confirmed in biomass

Dichlorobenzenes
Naphthalene
Acenapthene
Fluorene
Anthracene
Pyrene
Benz(a)anthracene
Chrysene

Compounds tentatively identified but not confirmed

C_3 Benzenes
Methyl Naphthalenes
Toluene
Ethyl Benzene
Xylenes

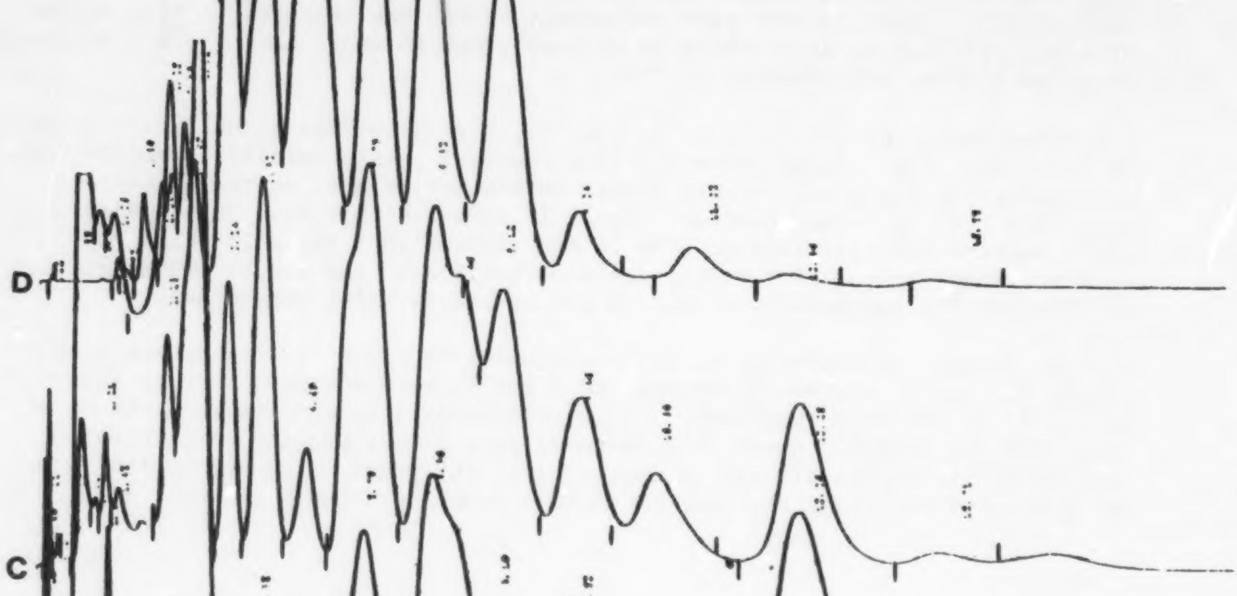

Figure 7. Gas chromatograph of polychlorinated biphenyls from electron capture analysis of algae grown on artificial substrates in the Delaware River during August 1979. Curve A = samples collected at ≈ri mi 110, Curve B = samples collected at ≈ri mi 108, Curve C = samples collected at ≈ri mi 100, Curve D = Aroclor 1254 used as standard.

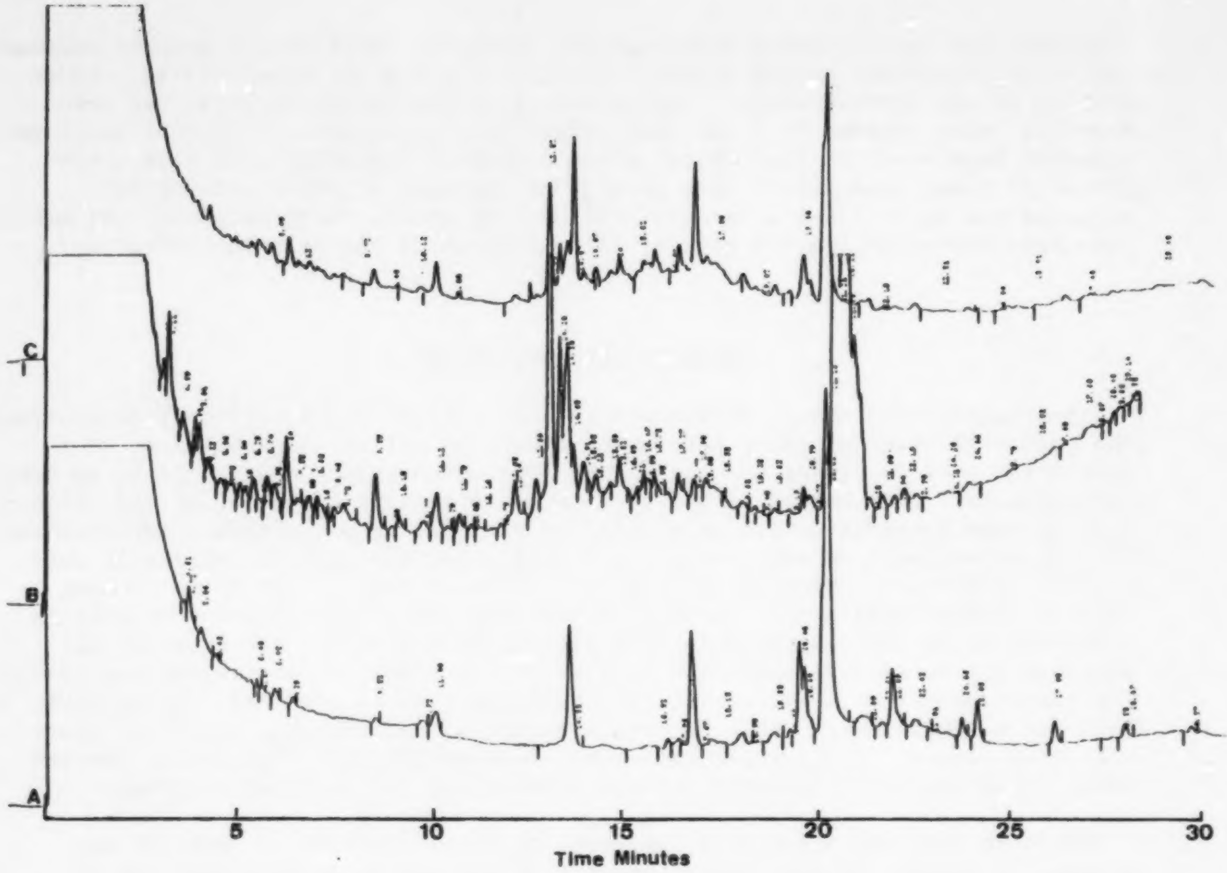

Figure 8. Gas chromatograph of organics determined by flame ionization, capillary column analysis of algae grown on artificial substrates in the Delaware River during August 1979.
Curve A = samples collected at ≈ri mi 110,
Curve B = samples collected at ≈ri mi 108,
Curve C = samples collected at ≈ri mi 100.

In addition to periphyton colonization, nonviable detritus and trapped sediment will also accumulate on the slides, although this did not appear to be a major problem in the above studies. The amount of nonbiological material has been found in other studies to range from slight to significant. Pollutant profiles obtained from areas of high detritus and suspended sediments will have a component of these materials. As a result, an estimate of direct biological accumulation by periphyton is more difficult to obtain in these areas. We are presently investigating the role of this material on the pollutant profile.

SUMMARY AND CONCLUSIONS

Investigations by other researchers and our current studies clearly demonstrate the potential value of using periphyton growth on artificial substrates as a monitoring tool for trace concentrations of heavy metals and organics in aqueous environments. This method offers both enhanced analytical detection and collection of time-integrated data of biologically available pollutants. Applications include assessments of point-source discharges for biologically available pollutants, effects of dilution and identification of chemical sinks in flowing systems, and as exploratory techniques for characterizing pollutants resulting from both point- and nonpoint sources present over wide areas. Although not suitable for certain research and applied problems because of limitations involving the mechanism of algal uptake and the complexity of chemical interactions in aqueous environments, this technique should be considered a powerful, cost-effective means of supplying considerable information both areally and through time, depending upon the nature of the problem and the sampling strategy.

Important research directions suggested by investigations to date include studies of changes in periphyton communities exposed to varying matrices of metal and organics; determining correlations between amounts accumulated and dry ash-free weight, chlorophyll or lipid content; investigation of seasonal effects on biological availability of metals and organics; and the use of this method in estuarine and coastal environments.

A research area of particular interest is the analysis for trace metals of unprocessed slides collected over the past 20 years as part of the ongoing diatometer program conducted by the Academy of Natural Sciences of Philadelphia. Slides are available from studies carried out on several major rivers in the United States; analyses of these slides could provide an historical perspective of these particular rivers.

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IN-SITU WATER QUALITY MONITORING USING TROUT IN THE U.K.

by

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The surveillance of water quality is a problem in common to many countries and whilst there may be geophysical and organisational differences between them, it is possible to observe similar requirements in the approach to a solution of that problem. The organisation of the water industry in the United Kingdom is such that the Water Research Centre acts as the central research agency for that industry. One aspect of in-situ biological monitoring receiving increased research effort is that of direct river abstraction surveillance using fish.

The technique employed uses noninvasive electrodes to detect bioelectric potentials generated by the fish and conducted through the water. Different signal components can be identified: respiration, heart action (e.c.g.), coughing, and physical motion. These signals are amplified, filtered, and processed to give three sets of data: respiratory rate, heart rate, and a total integrated signal called activity. Several systems are in operation at water treatment plants being managed by nonspecialist staff in some instances. The population of trout are changed regularly from test to stock tanks and are kept in an area isolated from natural daylight and excessive noise. To maintain the fish at low physiological levels, the test fish are unfed. The supervision of the fish population and associated equipment has been reduced to a once weekly visit of no more than 2 hours. By using up to 6 test fish, it is possible to improve the confidence in the reliability of response to the presence of toxic substances. It is not possible to produce a system in which the toxic substance can be identified nor quantified, but the strength of such a monitor lies in its ability to respond to a wide range of substances. Extensive toxicity tests have been conducted in the United Kingdom and Holland in an attempt to identify threshold levels to a variety of toxic substances and to seek out those that might affect man and not fish or vice versa. Typical results indicate the systems ability to detect toxic concentrations up to two orders of magnitude less than L.C. or L.D.50. The final system shows promise of acting as a 24-hour on-line monitor of toxicity at sublethal levels that may offer early warning of the presence of a hazard both to the water intake protection situation and to the biological population of the environment itself.

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*Mr. Solman was the only non-American to attend the biosensing conference and, while without a formal paper for presentation, kindly consented to speak extemporaneously and answer questions on matters relating to water-quality management in the United Kingdom. Subsequently, he prepared this precis of his remarks.

Working Panel Report 1

Biochemical Systems

Chairman: Mr. Theodore Major
Manager, Environmental Systems
Magnavox Corporation
Fort Wayne, Indiana

SUMMARY

The Biochemical Systems Working Panel included representatives from government agencies, universities, and industry. The composite experience of the panel in research development and operation of automated in-situ water quality sensors and monitoring systems was 105 person-years. (The panel members are listed below.)

The panel came away convinced that biosensors will fulfill a very substantial and complementary role in the overall water-quality monitoring mission for essentially three reasons.

1. Low cost, nonspecific pollution alarm systems are available for grab-sampler initiation.
2. Some low cost, highly specific pollutant sensors with great sensitivity are available for application (i.e., enzyme-based sensors).
3. Several new techniques are amenable to immediate prototype system fabrication and field test.

The working panel opened with a round-table discussion of the problems encountered by the workers and organizations involved in water-quality monitoring programs. This was followed by detailed technical discussions of specific biosensing techniques. The output of the discussions included the four broad recommendations discussed below.

RECOMMENDATION I

The panel selected the sensing techniques outlined below for additional development and funding. A priority rating established by its members and a rating was assigned to each technique. A total of eight was recommended.

No. 1 - Enzyme Probe Coupled to Ion-Selective Electrode

1. Applications - Detection of low concentrations of toxic compounds in drinking water, plant effluents. Alarm systems.

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2. State of the art

a. Status - research

b. Detection capabilities/limitation - 10^{-6} lowest likely detection limit. Absolute selectivity.

c. Expertise/sources

Dr. Shia S. Kuan
Department of Chemistry
University of New Orleans
New Orleans, LA 70122

d. Cost - $300,000 total to develop a field device.

3. Short-term research and development possibilities (less than 5 years) - Less than 5 years is more than enough for nitrate, nitrite detectors.

4. Long-term research and development possibilities (greater than 5 years) - See 3 above.

5. Remarks - Useful for monitoring drinking water. High priority.

No. 2 - Immobilized Enzyme Sensors

1. Applications - Detection of low concentrations of toxic materials; surveillance; monitoring.

2. State of the art

a. Status - Detectors for organophosphate and carbamate pesticide have been field tested and used routinely for hazard monitoring.

b. Detection capabilities/limitations - Extremely selective; good sensitivity; must find appropriate enzyme system.

c. Expertise/sources

Dr. Donald R. Sellers
Midwest Research Institute
425 Volker Boulevard
Kansas City, Mo. 64110

Dr. George G. Guilbalt
Department of Chemistry
University of New Orleans
New Orleans, La. 70122

d. Cost - $10,000 for cholinesterase inhibitor in any matrix; $100,000 for other enzymes/pollutants.

3. Short-term research and development possibilities (less than 5 years) - Short term.

4. Long-term research and development possibilities (greater than 5 years) - See 3 above.

5. Remarks - High priority.

No. 3 - Automated Bioluminescence

1. Applications - Effluent monitoring; surveillance; enforcement; hazardous materials spills.

2. State of the art

   a. Status - Manual test in production. Automated test at R&D level.

   b. Detection capabilities/limitations - Comparable to fish bioassay results. Bacteria often more sensitive than fish.

   c. Expertise/sources

      Dr. Anthony A. Bulich
      Beckman Microbics
      6200 El Camino Real
      Carlsbad, Ca. 92008

   d. Cost - $250,000

3. Short-term research and development possibilities (less than 5 years) - Short term.

4. Long-term research and development possibilities (greater than 5 years) - Long-range R&D for chronic test.

5. Remarks - High priority.

No. 4 - Enzyme Amplified Immuno Assay

1. Applications - Detection of low concentrations of target compounds.

2. Status of the art

   a. Status - Research and development

   b. Detection capabilities/limitations - Extremely sensitive and specific. Not a class detector.

   c. Expertise/sources

      Dr. Donald R. Sellers
      Midwest Research Institute
      425 Volker Boulevard
      Kansas City, Mo. 64110

d. Cost - $250,000 per system

3. Short-term research and development possibilities (less than 5 years) - Short term.

4. Long-term research and development possibilities (greater than 5 years) - See 3 above.

5. Remarks - High priority.

No. 5 - Heterogeneous Bioprobes

1. Applications - Detection of low concentrations of toxic compounds. Signal may be related to toxicity.

2. State of the art

a. Status - Methods developed; feasibility demonstrated. Not yet applied to environmental problems.

b. Detection capabilities/limitations - Dependent on toxic compound. Can measure enzyme at 10^{-11}M. Extreme specificity.

c. Expertise/sources

Dr. Mark E. Meyerhoff
University of Michigan
Department of Chemistry
Ann Arbor, Mich. 48109

Dr. Gary Rechnitz
University of Delaware

Dr. Robert Kobos
Virginia Commonwealth University

d. Cost - $100,000 per year

3. Short term research and development possibilities (less than 5 years) - Less than 5 years for development of laboratory-based equipment.

4. Long-term research and development possibilities (greater than 5 years) - See 3 above.

5. Remarks - Medium to high priority.

No. 6 - Fish Blood Chemistry

1. Applications - Evaluation of water quality. Measure effect on blood, liver, enzymes. Protection of particular species. Effluent toxicity. (Be alert for sublethal effects.)

2. State of the aart

a. Status - Research

b. Detection capabilities/limitations - Looking for sublethal effects, so probably parts per trillion. Currently manual method (as opposed to automated).

c. Expertise/sources

Dr. Dora R. May Passino
Great Lakes Fisheries Laboratory
U.S. Fish and Wildlife Service
Ann Arbor, Mich. 48105

Dr. Linwood Smith
College of Fisheries
University of Washington

A. Calabrese
National Marine Fisheries Service

3. Short-term research and development possibilities (greater than 5 years) - None

4. Long-term research and development possibilities (greater than 5 years) - Definitely long term.

5. Remarks - High to medium priority.

No. 7 - Bivalve Mollusk Blood Chemistries

1. Applications - Pollution and resource management

2. State of the art

a. Status - Research

b. Detection capabilities/limitations - Not necessary to put out field sensors; long lived; no seasonal changes. Catching organisms might change physiology.

c. Expertise/sources

Robert Dean
Dauphin Island Sea Lab
Dauphin Island, Ala. 36528

C. E. Epitanio
College of Marine Studies
University of Delaware

3. Short-term research and development possibilities (less than 5 years) - None

4. Long-term research and development possibilities (greater than 5 years) - Long term

5. Remarks - Medium to high priority

No. 8 - Microcalorimetry

1. Applications - Potential detector of toxic compounds. Based on measurement of the heat of metabolism of small organisms and changes in this heat as a result of stress.

2. State of the art

a. Status - Research instrument. Measurements made. Likely to remain a laboratory research instrument but potential for in-situ instrument has not been considered.

b. Detection capabilities/limitations - Not yet determined.

c. Expertise/sources

Edward Prosen
National Bureau of Standards

d. Cost - $100,000 identification of organism
$1,000,000 development of prototype in-situ sensor

3. Short-term research and development possibilities (less than 5 years - None

4. Long-term research and development possibilities (greater than 5 years) - Complete development would require more than 5 years.

5. Remarks - Medium priority. Usefulness for biological studies should be further demonstrated before consideration is given to modification for in-situ monitoring.

RECOMMENDATION II

It was recommended that a Federal Plan for Water-Quality Research Development and Monitoring be generated by the responsible agencies. This plan would serve four purposes.

1. Permit government program planners to allocate their resources in a systematic manner.

2. Permit industry to identify planned and budgeted programs and markets. (This is a mandatory step prior to allocation of internal research and development funds.)

3. Allow universities to allocate their resources to "real" water-quality monitoring problems.

4. Allow government agency researchers to integrate their activities with the Federal Plan. As an example, the Federal Plan for Ocean Pollution Research, Development, and Monitoring could form an integral element of the overall Federal Plan for Water Quality Research Development and Monitoring. (Note that integration of developmental programs was recommended by the first water-quality sensing workshop *Automated In-Situ Water Quality Sensor Workshop*, held in Las Vegas during the period February 14-16, 1978 and sponsored by the Environmental Protection Agency, the National Oceanic and Atmospheric Administration, and the Interagency Working Group on Satellite Data Collection Systems.)

Comments on the Federal Plan for Water-Quality Research Development and Monitoring

The Federal Plan must be an interagency one that integrates the responsibilities of the several federal agencies involved, such as the Environmental Protection Agency, the National Oceanic and Atmospheric Administration, the U.S. Coast Guard, and the U.S. Geological Survey, the U.S. Army Corps of Engineers, and the Department of Agriculture, as well as regional state and local governments. While the overall plan should be comprehensive, its initiation should be accomplished on the basis of a demonstration project where the research, development, and field activities could be accomplished on a practical basis. The BLACK CREEK project sponsored by EPA in Allen County, Indiana, is an excellent example of such a project dealing with agriculture pollution.

The plan must provide for effective utilization of the data acquired. This provision would include five actions.

1. Input to polluters for immediate response
    - Industries (stop the spilling and dumping)
    - Transportation
    - Municipal waste water treatment authorities

2. Input to pollution abatement organizations
    - Contractors
    - U.S. Coast Guard
    - Municipal organizations

3. Public appraisal (alarms, trends)
    - Drinking water
    - Contact sports
    - Fish consumption
    - Ecological health of water bodies

4. Enforcement organizations
    - EPA
    - USCG
    - State and municipal organizations

5. Legislators
    - Local
    - State
    - Federal

Data Distribution

Standards must be set for data coding for computer-to-computer interaction. The NOAA/NESS data communications scheme for the GOES satellite must be incorporated into the system and could serve as the standard. The data rate is slow enough to be compatible with dial-up telephone systems similar to those used by the National Weather Service AFOS system. As an example, a dial-up data distribution system installed in a river monitoring system could be used to warn municipal water treatment operators of impending slugs of pollutants coming downstream. Similarly, standards must be set for data accuracy (quality assurance).

By setting such standards, the Water-Quality Data Distribution System would grow as dictated by priority requirements and demonstration projects.

Monitoring System Automation

The objective of automation is to save money and to acquire the warning data required to protect the public and ecologic health. Accordingly, automatic water-quality monitoring stations would impinge in four basic areas.

1. Drinking water processing plant inputs and outputs
2. Industrial plant outfalls and streams
3. Waterwater treatment plant outfalls
4. Remote inaccessible locations

The Automatic Water Quality Monitoring stations should be equipped only to sense those pollutant parameters which will be required for immediate response. If this is not the case, the alarm sensor may trigger a sample acquisition for subsequent laboratory analysis and reporting.

The Automatic Water Quality Monitoring stations may utilize three techniques to provide alarm sensors.

1. Biologic sensors (toxicity)
   Fish and mollusks - stress detection
   Bacteriums - luninescent stress

2. Traditional sensors
   Conductivity
   pH
   Dissolved Oxygen
   Temperature

3. Multiparameter analyzers (automated)
   Heavy metals - energy dispersive x-ray
   Pesticides (organic) - gas chromagraphs

NOTE

> The primary application for multiparameter analyzers is in the inputs and outputs of municipal potable and waste water processing plants.

The working panel observed that much of this technology is already available but not generally utilized. The Ohio River Sanitary Commission (ORSANCO) is a leader in this respect; however, their annual equipment procurement budget is limited to approximately $100,000 per year.

The enzyme sensors currently in early stages of development show great promise in providing both the specificity and the sensitivity required to perform in automatic alarm systems as well as in laboratory measurement systems.

Manual Monitoring Applications

Manual analysis (i.e., in the laboratory) is particularly useful and applicable in the detection of chronic subtoxic level pollution. This procedure is necessary to predict water-quality trends and to establish the lower levels of permissible pollution concentration. Another very valuable product of laboratory analysis is the establishment of the sensitivity levels required of field instrumentation such as bioprobes and specific ion sensors.

The tecniques for manual pollution trend monitoring may include the use of three bioaccumulators, that is,

1. Algae
2. Bivalves (invertebrates)
3. Fish

The monitoring process may provide for natural acquisition of the organisms in the monitoring site or for the caging and implantation of control organisms. The organisms are exposed for the desired period and returned to the laboratory for analysis using traditional instrumentation.

RECOMMENDATION III

A third recommendation concerned support by the federal government for the development of automatic in-situ water-quality monitoring systems. The key element of systems is the water-quality sensor or transducer. This support is necessary since a federal, state, or municipal agency is the primary customer for automated in-situ water-quality monitoring systems. These automated systems are required to

1. Save monitoring cost and manpower,
2. Provide near-real-time data as required for corrective action (alarms),
3. Provide a measure of pollutant volume rate of travel dispersion, etc., and
4. Trigger sample acquisition devices as required for enforcement proceedings.

RECOMMENDATION IV

Lastly, it was recommended that federal, industrial, and university organizations be appraised that the research and development activity in the field of biosensing and ion-selection electrodes in Europe and the U.K. is proceeding at an accelerating pace. (In the United States, the effort has been confined to a relatively small number of universities and research institutes.)

Members

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Working Panel Report 2

Bioaccumulation

Chairman: Dr. Andrew Robertson
Head, Chemistry-Biology Group
NOAA/GLERL
Ann Arbor, Michigan

Panel 2 was convened to discuss the use of aquatic organisms as accumulators or samplers of contaminants. The panel attempted to identify the most promising areas for development and use of such bioaccumulators. A summary of findings follows, particularly in terms of development. In addition, a brief outline of the bases used by the panel to arrive at these decisions is presented. (Panel members are listed below.)

Potential Uses for Bioaccumulators

As a first step in identifying the areas of most promise for developing bioaccumulation techniques, the panel identified the major uses that could be made of such measurements. This approach involved developing a list of the ways in which these measurements might aid in controlling water-quality degradation. Five such considerations are discussed below.

1. As a screening technique. Organisms can be analyzed to determine their levels of bioaccumulation for a broad spectrum of contaminants. These analyses can aid in identifying which contaminants are found in a specific aquatic environment at substantial levels and those that warrant further study as potential problems.

2. As an early warning. Bioaccumulation may be used as a method to identify when contaminants are building to concentrations that may cause substantial deleterious environmental effects.

3. As a means to characterize the seasonal and geographical distribution of contaminants. Comparative measurements of bioaccumulation at various times and places may provide valuable information on the seasonal and geographical distributions of contaminants and thus provide assistance in understanding the dynamics and cycling of such substances.

4. As a regulatory aid. In many cases, regulatory agencies have set objectives or standards designating the concentrations that specific contaminants should not exceed in the environment. These are sometimes expressed as concentrations allowable in specific organisms, often fish. Thus, bioaccumulation measurements can give direct information on whether these control concentrations are being exceeded. Standards are also sometimes expressed as concentrations in a given volume of water. If the relation between concentration in the bioaccumulating organism to that in water is known, bioaccumulation measurements can be a convenient

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and often less expensive way to determine whether such water concentrations are being exceeded.

5. As indicators of sources of pollutants. Bioaccumulators may be placed at various locations throughout an area where excessive containment concentrations may pass. By comparing the bioaccumulation at such sites, it would often be possible to identify the containment source. This technique can be especially valuable when contaminant releases occur in locations or at times when conventional sampling would not detect them.

General Categories of Bioaccumulation Techniques

The panel concluded that the use of organisms as samplers or bioaccumulators of contaminants can be usefully divided into three general categories.

The first category includes the most straightforward techniques in which naturally occurring organisms are collected in their native environment and analyzed for their accumulated pollutants. In this category, nothing is known about the past history of the organisms analyzed, so interpretation of the results can be difficult and conclusions ambiguous.

In the second category, organisms of known history are exposed in the laboratory to samples of the environment to be tested. After a set period of exposure in this laboratory setting, the organisms are analyzed to determine how much of the contaminant or contaminants of interest have been accumulated.

The third category is similar to the second in that the experimenter places organisms of known history in contact with the suspected polluted environment. However, in this category the organisms are exposed in the natural environment rather than in the laboratory. After a specific period of time, the contaminant load is measured.

A great deal of attention in pollution research has been given to the development of techniques for categories one and two. A number of methods are well established especially in category one.

For the third category, however, while several specific techniques have been used, development of the approach has been so limited that its applicability is still largely unproven. The panel believes nevertheless that techniques from this category hold great promise for aiding water-quality studies and regulation. Consequently, they decided to limit their recommendations to identifying promising areas for development under this category only.

General Points

Before proceeding with recommendations, the panel developed a set of seven general points that apply to all the recommended techniques.

1. Techniques must be such that adequate material for analysis of the contaminant or contaminants of interest can be obtained.

2. Techniques should provide bioaccumulation concentrations that can be related to ecosystem effects or studies providing this information should be carried out.

3. Values obtained by any technique should be relatable to environmental concentrations of the contaminant or studies to provide this information should be conducted.

4. As different organisms concentrate contaminants from different compartments of the environment, it is necessary to determine what compartment the organism in use is really sampling.

5. Techniques must be matched to the contaminant or contaminants of interest since different species bioaccumulate the same pollutant to very different degrees.

6. Similarly, the same organism bioaccumulates different contaminants to very different extents.

7. It is not enough to simply measure bioaccumulation in order to further water-quality management. Relating the measured bioaccumulation values to economic consequences is very important, as is the interpretation of the results in terms of regulatory needs.

Specific Recommendations for Development of Bioaccumulation Techniques

The panel concluded that there are two basic ways for placing organisms at a specific location in the environment and measuring the amount of contaminants they bioaccumulate.

The more conventional method calls for putting determined numbers and types of organisms at a specific location and then retrieving them after a certain length of time and measuring their levels of bioaccumulation. The first three recommendations below relate to this subcategory.

The second method is somewhat less conventional but holds substantial promise in the panel's view. This calls for placing a structure that will attract, or cause settling of, certain types of organisms in a designated location in the environment. After a certain length of time, the structure with the organisms that have settled and grown on it is retrieved and the bioaccumulations of the contaminant or contaminants of interest are determined. The final three recommendations apply to this second method.

Recommendations

Application No. 1 - Biomonitoring Chambers

1. State of the art

a. Status - Membrane biomonitoring chambers are in limited production; chambers with other types are in the research stage.

b. Detection capabilities and limitations - These chambers have great applicability in determining bioaccumulation from the dissolved phase; they do, however, usually exclude particulate material. Fouling of the chambers may cause problems as well as the fact that the organisms themselves restrict current flow and may alter other environmental conditions.

c. Sources of expertise - Dr. H. Schlichting (BioControl Co.); Dr. McFetters, Montana State University; Dr. T. Smayda, University of Rhode Island

2. Short-term research and development possibilities (less than 3 years) - There is a need to reduce fouling problems, possibly by altering the confining material. Also, research should be continued on improving the design and materials of biomonitoring chambers and on defining the applicability of the technique.

3. Long-term research and development possibilities (greater than 5 years) - Long-term research and development is needed to automate water-quality measurements inside and outside the chambers and to relate bioaccumulation in organisms in the chambers to environmental effects.

4. Remarks - These chambers may be useful as a legally valid means to establish the source of a pollutant. There are a variety of promising areas for development of these chambers; thus, the panel rates them a high priority for further development.

Application No. 2 - Biomonitoring by Sessile Filter Feeders

1. State of the art

a. Status - There are several operational systems using sessile filter feeders to determine bioaccumulation (e.g., Mussel Watch). Validation of the meaning of the results from these systems is still required.

b. Detection capabilities and limitations - Filter feeders bioaccumulate particle-transported contaminants; they are much less likely to concentrate contaminants from the dissolved phase. These organisms may have avoidance behavior for high concentrations of certain contaminants that would cause them not to accumulate the pollutant when it is present in such contentrations.

c. Sources of expertise - Scripps Institute of Oceanography (Dr. E. Goldberg); NERC Laboratory (Plymouth, England); NMFS Laboratory (Milford, Conn.)

3. Short-term research and development possibilities (less than 3 years) - Further research and development is required to determine what species to use for specific purposes and how to improve standardization of this technique. Further development is also needed to improve the retrieval system for certain applications.

4. Long-term research and development possibilities (greater than 5 years) - Long-term research is needed to improve understanding of the meaning of measured levels of bioaccumulation, especially with regard to effects on important resources.

5. Remarks - This method has high potential for combining measurements of bioaccumulation with determination of contaminant effects on the test organisms. The panel rates this technique as having high potential for further development.

Application No. 3 - Biomonitoring by Confined Deposit Feeders

1. State of the art

a. Status - The panel had no knowledge of any efforts to use confined deposit feeders as bioaccumulators. However, because sediments are often the ultimate sink for contaminants, development of such a system was considered very desirable.

b. Detection capabilities and limitations - Such a system should have the capacity to monitor a broad spectrum of sedimentary contaminants. Possible difficulties include the effects of confinement on bioaccumulation, fouling interferences, and possible escape of the bioaccumulating organisms.

c. Source of expertise - None known

2. Short-term research and development possibilities (less than 3 years) - The first priority in the exploitation of this technique is to develop a prototype system.

3. Long-term research and development possibilities (greater than 5 years) - Long-term needs cannot be identified until development of this technique has been initiated.

4. Remarks - The panel felt that a technique to monitor pollutant bioaccumulation in the sediments is very badly needed, and so, even though the cost of development of a system to use confined deposit feeders may be quite high, they rated the development of this technique as having high priority.

Application No. 4 - Artificial Substrates for Freshwater Periphyton (Periphytometer)

1. State of the art

a. Status - The biological aspects of colonization of artificial substrates is quite well understood. The instrument to perform this function is in the research state. (See the paper by Dr. Friant published elsewhere in this volume.)

b. Detection capabilities and limitations - This technique will allow measurement of the bioaccumulation of a broad spectrum of contaminants. However, the technique is not suitable for strongly polar substances or those that are easily metabolized by the organisms involved.

c. Source of expertise - Philadelphia Academy of Sciences (Dr. Friant)

2. Short-term research and development possibilities (less than 3 years) - Short-term research and development is needed on seasonal effects on biological availability of pollutants, on evolution of sediment contributons to contaminant accumulations on periphytometers, on establishing correlations between bioaccumulation and ash-free periphyton weight, and on how communities change on exposure to contaminants.

3. Long-term research and development possibilities (greater than 5 years) - Long-term research and development is needed to improve the design of the periphytometer and to automate the instrument for in-situ analysis of contaminants.

4. Remarks - This technique would be especially valuable if biological measurements of diversity and species abundance were made on the same samples so that bioaccumulation values can be correlated with these biological properties. The panel rates this technique as having a high priority for further development.

Application No. 5 - Artificial Substrates for Marine Periphyton

1. State of the art

a. Status - The same instrument as used in the preceding freshwater application can be used in marine waters. However, it has received little use in these environments so far and may require some modification.

b. Detection capabilities and limitations - Same as for freshwater application.

c. Sources of expertise - Philadelphia Academy of Sciences

2. Short-term research and development possibilities (less than 3 years - Short-term research and development is needed to adapt this instrument for for marine environments and to develop the background to interpret the results obtained.

3. Long-term research and development possibilities (greater than 5 years) - Same as the freshwater instrument.

4. Remarks - The use of this instrument may present more difficulties in the marine environment than in freshwater due to heavier deposition of inorganic material and to settling of a greater variety or organisms. Both of these effects may make interpretation of the results more difficult. Because of these potential difficulties, the panel rates this technique as having medium priority for further development.

Application No. 6 - Artificial Substrate for Benthic Recolonization

1. State of the art

a. Status - The study of benthic recolonization is quite well advanced. However, to the panel's knowledge, no artificial substrates have been developed to take advantage of this for bioaccumulation studies.

b. Detection capabilities and limitations - This technique would allow measurement of bioaccumulation by deposit feeders. One limitation is that retrieval may require divers.

c. Sources of expertise - Virginia Institute of Marine Sciences, and the U.S. Environmental Protection Agency at Gulf Breeze, Florida.

2. Short-term research and development possibilities (less than 3 years) - Existing methods for studying recolonization need to be adapted for artificial substrates to look at bioaccumulation.

3. Long-term research and development possibilties (greater than 5 years) - None identified

4. Remarks - This type of approach may be the most promising way to monitor bioaccumulation of pollutants in the deep sea, and would be particularly useful for studying the recovery of communities and the relation of this to bioaccumulation. The panel rates this technique as having medium priority for further development.

Members

Working Panel 2 - Bioaccumulation

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Working Panel Report 3

Organism Behavioral Responses

Chairman: Mr. Richard W. Paulson
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Water Resources Division
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The panel concluded that there were two objectives in studying orgnism behavioral response for application as in-situ sensors. (The names and associations of the panel members are listed below.)

Objective No. 1 - To develop a technique to measure broad-scale variations in water quality.

Objective No. 2 - To better understand the way organisms sense water quality in order to develop sensors that use the same technique.

The panel also concluded that the following 9 criteria should be considered when organism behavioral response is used as an in-situ sensor monitoring mode.

Criterion No. 1 - A real-world approach should be used when organism behavioral response is used in in-situ monitoring, i.e., unnecessary complexity is to be avoided.

Criterion No. 2 - Common, easily available, endemic, and well-known species should be used where possible. Behavioral response of an organism is important when it deviates from the normal range, and it is imperative that the organism be a common one so that its normal response is understood.

Criterion No. 3 - The organism should operate at ambient conditions of the water body under measurement in order to maximize the efficiency of station operation.

Criterion No. 4 - The organism should not be so delicate that it cannot tolerate minor perturbations, nor so rugged that it does not respond to large perturbations.

Criterion No. 5 - The organism should respond to water quality in a way that can be observed, measured, and monitored in a noninvasive way.

Criterion No. 6 - The expected range of water quality should subject the organism to sublethal stress only.

Criterion No. 7 - The organism should come to equilibrium in the test environment within a reasonable length of time, such as 24 hours. The organism population at a monitoring location should be managed so that part of the population should be in reserve, part coming to equilibrium, with part yet under test.

Criterion No. 8 - The organism should be used as a management tool in the sense that behavior outside the individual organism's normal range should be used as a key for more detailed and conventional water sampling and analysis.

Criterion No. 9 - Organisms will not be completely predictable in behavior and will respond to condition with some uncertainty, i.e., false alarms can be expected.

Conclusions and Recommendations

Fish probably offer the most immediate means of in-situ monitoring using behavioral response. It is appropriate to begin using fish in operational stations as is done in the United Kingdom and Europe.

Behavior in invertebrates has not been as studied, as with fish, but there are a number of groups that offer possibilities as in-situ monitors. Crustaceans, including crabs, lobsters, crayfish, and shrimp have a number of behavioral patterns that could be adapted to monitoring, but would require a costlier and longer developmental program than for fish.

In those groups that have been studied (e.g., crabs and lobsters), the responses to dissolved substances have indicated that invertebrate chemosensors are extremely sensitive and thus offer potential for in-situ monitoring. Research and development costs and time, though greater for invertebrates, may be warranted for some species because of their levels of sensitivity.

Some molluscan behavioral responses, e.g., valve (shell) openings and closings (pumping behavior), have been used successfully as physiological monitors and are also quite sensitive to water quality changes. The valves can be hooked to hymographs or printers of some sort to facilitate automation. The organisms feed themselves and require little care.

Other bivalve species such as scallops employ swimming behavior to escape predators. This reaction can be tested by dropping starfish extract into the water. A large number of clam species are capable of leaping movements, whereby the foot is extended and curled under the body and then forcibly straightened. This movement propels the clam upward away from the bottom. Again, this can be elicited by dropping extract of predator species. Deviation of the swimming behavior may be an indication of the organism's ability to cope with water-quality variations.

These behavioral responses indicate a very sensitive chemosensory mechanism which might ultimately be tapped for in-situ monitoring.

The scope of organisms considered was limited by the small number of participants on the panel and their background and experience as biologists. Thus, this discourse should not be considered exhaustive.

The panel's recommendations follow.

Recommendations

Application No. 1 - Fish Behavioral Response

1. State of the art

a. Status - Instrumented automated monitoring systems exist to collect physiological and rheotactic data. Laboratory systems are in use in the U.S., while operational capabilities exist in the U.K., Holland, and West Germany.

b. Detection capabilities and limitations - Capable of detecting toxins to levels 10^2 lower than LC_{50}. Some substances toxic to fish are not toxic to man and the opposite is true as well.

c. Sources and expertise

U.S.A.	Dr. John Cairnes University Center for Environmental Studies Virginia Polytechnic Institute Blacksburg, Va.
	Dr. Donald E. Wohlschlag Institute of Marine Science Port Aransas Marine Laboratory University of Texas Austin, Texas
U.K.	Arthur Solman Water Research Centre Medmenham Laboratory Buckinghamshire
Holland (K.I.W.A.)	Dr. Poels
South Africa	Morgan
W. Germany	Kerren Aquatest and Zippe Monitor

d. Costs and times

Developmental: Greater than $100,000 to develop improved sensor capability.

Prototype: Greater than $20,000

Production: Certain European systems are presently available for $15,000 to $20,000.

2. Short-term research and development possibilities (less than 3 years) - New transducers to improve signal separation techniques and pattern recognition techniques need to be developed. This approach, however, should not inhibit operational use of this methodology in the short term.

3. Long-term research and development possibilities (greater than 5 years) - There is a need for development of flexible monitoring systems that can support a variety of organisms, a variety of sensors, ongoing testing, and a better data base of information for analysis.

4. Remarks - A continuing dialogue between workers active in this field and agencies responsible for environmental monitoring is definitely needed.

Applicaation No. 2 - Invertebrates Such as Blue Crab (Callinectes Sapidus)

1. State of the aart

a. Status - Experimental. Feeding behavior such as antenna flicking and gill bailing are being studied in detail.

b. Detection capabilities and limitations - Blue crabs can respond to food materials at a level of 10^{-15} gram/liter. They respond with changes in the rate of antenna flicking and gill-bailing behavior. It may require some expertise to recognize and quantify behavioral response automatically.

c. Sources of expertise

W. H. Pearson, Batelle institute

Bori Olla, NMFS, Sandy Hook

d. Costs and times

Developmental: On the order of 2 years and $100,000.

Prototype: On the order of $50,000

Production: Difficult to assess

2. Short-term research and development possibilities (less than 3 years) - Much basic research is required but there is a good chance that responsive sensors could be developed within 3 years.

3. Long-term research and development possibilities (greater than 5 years) - Difficult to assess but automated systems to monitor invertebrates could be ready in 5 years.

4. Remarks - The risk is great as are the potential benefits.

Application No. 3 - Molluscs Such as Eastern Oyster (Hassastrea Virginica)

1. State of the art

a. Status - Pumping behavior often is used as a physiological indicator, although not necessarily as a measure of toxics.

b. Detection capabilities and limitations - Not well known beyond its ability to detect changes in temperature, salinity, and turbidity.

c. Sources of expertise

H. H. Haskes - Rutgers

H. Hido - U. of Maine

R. Hillman - Battelle

d. Costs and times

Developmental: $100,000

Prototype: 2 years on the order of $75,000

Production: Difficult to assess

2. Short-term research and development possibilities (less than 3 years) - Can probably be automated relatively easily. Research really required to assess reaction of organism to various toxics.

3. Long-term research and development possibilities (greater than 5 years) - Once the toxic reaction of the organism is known, the automation will be simple.

4. Remarks - With regard to freshwater molluscs, not much is known. On the other hand, a considerably larger body of knowledge is at hand regarding marine molluscs (those sold commercially).

Application No. 4 - Zooplankton (diurnal movements with copepods and arrow worms, for example

1. State of the art

a. Status - Much is known about vertical movements corresponding to day/night periods (photoresponse). These vertical migrations might offer possibilities for study.

b. Detection capabilities and limitations - Limitations involve short life cycles. From species to species, there is a variability in behavior patterns. However, pure cultures are easy to raise.

c. Sources of expertise

C. I. Gibson
Battelle
Marine Research Laboratory
Sequim, Washington

S. Herman
Lehigh University
Bethlehem, Penna.

d. Costs and times

Developmental: Given $300,000, 3 to 4 years.

Prototype: At this time, neither is specifiable.

Production: At this time, neither is specifiable.

3. Short-term research and development possibilities (less than 3 years) - Short-term research could be aimed at defining migration patterns for representative species and assessing species ability to detect pollutants (and alter behavior).

4. Long-term research and development possibilities (greater than 5 years) - Longer term programs would probably be required to use this approach feasibly.

5. Remarks - The probability of success using this technique is difficult to forecast.

Members

Working Panel 3 - Organism Behavioral Responses

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Working Panel Report 4

Traditional Biological Measurements

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Army Corps of Engineers
Water Resources Support Center
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Cochairman: John Bushman
Army Corps of Engineers
Washington, D.C.

CONCLUSIONS AND RECOMMENDATIONS

Chlorophyll is a basic biological parameter whose measurement is useful for water quality monitoring purposes. However, further refinement of classical chlorophyll measurement techniques is probably not particularly fruitful.

New techniques such as in-vivo chlorophyll fluorescence measurements have a high potential for automated in-situ water quality monitoring.

Summary

Many agencies have expressed a need to increase the volume of biological samples processed. Traditional biological techniques make this an extremely labor-intensive task. Increasing the speed of collecting and processing are of great concern. URI's research into the use of image processing and pattern recognition for zooplankton analysis represent a major step toward automation. VPI has been using optical image processing and pattern recognition to analyze diatoms. The panel recommends that for automated processing and analysis of biological samples:

- Research into image processing and pattern recognition be continued.
- Research into in-situ photographic teachniques be extended.
- Long-term research into potential for telemetry of biological data be initiated.
- Strategy of oceanographic and limnological sampling plans be prepared.
- Encourage interaction with mapping of ocean productivity by remote (satellite) sensing techniques.

Recommendations for Traditional Biological Measurements

Chlorophyll

1. State of the Art:

a. Status - Chlorophyll measurements have been used for decades as estimates of algal standing crop (biomass) relative productivity, and trophic state. Chlorophyll pigment is closely coupled with photosynthesis, so the measurement is very useful for productivity--or eutrophication-related purposes. The most traditional method of measurement is spectrophotometric determination of absorbance on extracted samples (i.e., in vitro), although in-vitro measurements are also now used widely. In-vitro methods are relatively well standardized and commonly employed in oceanographic areas and limnological research. In-vivo (not extracted) chlorophyll fluorescence measurements are more recent, but are used widely to investigate vertical and horizontal distribution of phytoplankton. DCMV-enhanced in-vivo chlorophyll fluorescence is a relatively new modification that significantly improves the information content of chlorophyll measurement by providing an indication of the physiological status of the phytoplankton assemblage sampled, in addition to the algae biomass present.

b. Detection Capabilities/Limitations - Chlorophyll measurements are sensitive over a broad range of concentrations, particularly with fluorometry. Detection limits are not really a problem.

Primary limitations stem from inherent variability of chlorophyll: plant carbon and fluorescence; chlorophyll relationships. Historical methodological problems with in-vitro methods are (1) differentiation of absorption (fluorescence emission peaks in complex pigment mixtures), (2) extraction efficiency, and (3) presence of phaeopigments. Phaeopigment concentrations can be estimated and corrected. In-vitro and in-vivo fluorescence measurements must be related to spectrophotometric determinations to estimate chlorophyll concentrations. Chlorophyll measurement provides a good relative indication of algal biomass rather than an exact determination.

c. Sources of Expertise - Numerous chlorophyll methodologies are in common use in oceanographic/limnological research and water quality monitoring.

d. Costs and times - Traditional in-vitro methods can probably not be improved significantly. In-vivo fluorescence methods, particularly DCMV-enhanced fluorescence, have potential for further development. DCMV method requires more extensive evaluation and automation. Automation can probably be accomplished with off-the-shelf components (continuous-flow fluorometer, automatic injection system, timing devices, and data storage systems). The time required for evaluation of this method including prototype development and field testing is about three years at a cost of approximately $100,000/yr.

2. Short-term research and development possibilities (less than 3 years) - Requirements for short-term R&D are to further evaluate the DCMV-method, including development of a prototype automated system and a field verification project. The entire program can be accomplished within a 3 year time frame.

3. Long-term research and development possibilities (greater than 5 years) - The long term goal would be to interface the system with other automated water quality monitoring systems (physical-chemical sensors, particle counters, image processing, auto analyzers, etc.).

4. Applications - The application of the system would be in continuous monitoring and/or surveying of (1) algal standing crop, (2) relative productivity/tropic state, (3) algal physiological condition, and (4) changes in the above due to nutrient/pollutant/toxicant loading. In addition, continuous flow fluorometry can provide the ground truth for chlorophyll measurement so that a comparison/evaluation of remotely-sensed (satellite) chlorophyll data can be made.

5. Data Handling - Extent of data handling required depends on the apoplication. It can range from a simple chart recorder for survey work to a data logger/computer for continuous monitoring. Time series analyses is perhaps the best for large data sets.

6. Summary

a. Further refinement of classical chlorophyll measurement techniques is probably not required.

b. In-vivo chlorophyll fluorescence measurements have high potential for automated water quality monitoring.

c. DCMV-enhanced in-vivo chlorophyll fluorescence method provides information on the amount of algal biomass present and algal physiological condition. The method requires additional evaluation but it potentially can also be automated.

Recommendations for Computerized Processing of Plankton Samples

1. State of the art

a. Status: A developmental/engineering model has been constructed, programmed, and tested at the University of Rhode Island. Large scale sampling programs are in progress which require automated analyses.

b. Detection Capabilities and Liimitations: Major taxonomic groups in the zooplankton have been measured, recognized, and counted (e.g., copepods, cladocera, chactognaths, fish eggs, and euphousids); more detailed classification appears possible upon solution of technical problems (imaging, specimen orientation).

c. Sources of Expertise:

University of Rhode Island, NOAA/Narragansett -
Poularikas, Jeffres, Oherman, Mouser
NOAA/Miami - Ortner
WHOI - Wiebe
Scripps - Burg
VPI - Cairns

d. Costs and Times:

Developmental: completed

Prototype: 2-3 yeaars, 60-70K/unit (hardware costs)

Production: no estimate at present

2. Short Term Research and Development Possibilities (less than 3 years): Further testing of algorithms; optimization of computer space and speed; refinement of imaging and specimen orientation techniques; field testing of prototype in other laboratories; and combining statistical and syntactic programming.

3. Long Term Research and Development Possibilities (greater than 5 years):

- o Incorporation of operation interaction - machine learning algorithms
- o Applications to phytoplankton
- o Photographic archiving of plankton samples
- o Telemetry of data -- for making real-time decisions in the conduct of oceanographic sampling programs (vs. grid and transect approach)
- o Interaction with mapping of ocean productivity using remote sensing techniques
- o Fisheries optimization dynamics -- determination of growth ratios (fish-scale image analysis), nutritional availabilities and requirements for sustained growth; and recruitment rates and success.

4. Remarks

a. Applicability to analysis of ecosystem processes: The state and rate of change in relation to environmental variation, both natural and man-induced (e.g. plankton distribution around and in pollution fields arriving from wastewater discharges and petroleum spills)

b. Sensor need: A facility for analysis should be available at all levels, from local to international.

c. Number of sensors: Regional pattern recognition centers should be established for high speed processing of plankton samples; the facilities would also operate on integrated data management system

d. Likelihood of success: Identification, enumeration, and size-frequency distributions have been made by computerized means of pattern recognition; the need for engineering refinement in certain areas is now obvious.

e. Cost effectiveness: A comparison of manual and automated means of zooplankton sample analysis (counting, size-frequency distribution) shared time saving of 60-80% by automated procedures.

f. Skill level: A skilled operator capable of traditional zooplankton analysis is essential; also some facility with the management of mid-size computer programs would be desirable. A Bachelor of Science degree in natural science, electrical engineering, or computer science should be required, plus 6 months to 2 years practical experience.

Members

Working Panel 4 - Traditional Biological Measurements

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